MECHANISM OF ACTION

OF

STEROID HORMONES

A Thesis presented for the Degree

of

DOCTOR OF PHILOSOPHY

by

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SECTION I

GENERAL INTRODUCTION

Fig. 1. 1. Uterus from an ovariectomised untreated rat (left) and from a similar animal which had received 10 μg oestradiol subcutaneously daily for 3 days (right).

These investigations will deal mainly with oestrogenic steroid hormones, except in SECTION II where work with adreno-corticosteroids is reported.

Since the isolation some thirty years ago of steroids which in minute amounts produce oestrus in animals (Doisy, Veler & Thayer, 1929; Butenandt, 1929; Marrian, 1930), great interest has been directed towards an explanation for their profound biological effects. These effects of oestrogens in the whole animal, both gross and microscopic, have been the subject of numerous qualitative and quantitative descriptions. The anatomical changes produced in the uterus of the immature or ovariectomised rat (Bulbring & Burn, 1935; Lawson, Heller, Golden & Severingham, 1941; Evan, Varney & Keoh, 1941, see Fig. I. 1), in the chick oviduct (Hertz, 1948) and in the mammary gland (see Burrows, 1949a) in response to the administration of minute doses of oestrogen, are well recognised and easily discernible. In addition to the gross alterations, there are changes in the cellular population of some tissues particularly of the vaginal epithelium (Stockard & Papanicolaou, 1917; Allen & Doisy, 1923).

Those tissues which respond in a striking manner to a hormone are commonly termed 'target' organs of that hormone. It is impossible at present to define just

what differentiates, at the cellular level, a target organ from other tissues of the body. There is evidence that enzymes from two tissues though catalysing the same reaction may be distinctly different and subjected to different degrees of hormonal control. Henion & Sutherland (1957) have shown, for example, that the phosphorylase of liver responds to glucagon, whereas the phosphorylase of muscle does not.

In considering the types of mechanism by which oestrogens may exert their biological effects, several general approaches have been made. The most common working basis originates from the theory of Green (1941) which states that "any substance which in trace amount produces biological effects does so either by participating in, or by specifically affecting some enzyme systems." This hypothesis predicts that:

(a) the hormone may regulate the activity of an enzyme system by controlling inhibitors or activators.

(b) the hormone may function as an essential component of the enzyme system such as a coenzyme.

(c) the hormone may alter the rate at which new enzyme molecules are produced from simpler precursors.

In addition to this 'trace substance-enzyme' theory, Hechter (1955) proposes that the hormone may change

the permeability of the cell membrane or the membrane around one of the subcellular structures such as the mitochondria and thus make substrate or cofactor molecules more readily available to enzymes.

There are at the present time few direct experimental data bearing upon the effects of oestrogen at physiological concentration on cell permeability. Technical difficulty is the main obstacle to a direct approach to the study of this problem of hormone action at the cellular level (see Hechter, 1955). On the other hand, considerable attention has been paid to the concept that hormones exert their effects by acting in some way upon enzyme systems.

The first of the two major investigations of the action of hormones on enzymes involves the comparison of the in vitro metabolism of preparations of 'target' tissues removed from ovariectomised animals with those taken from similar animals treated with physiological doses of oestrogen. In an important series of experiments, Mueller, Herranen & Jervell (1958) showed that in the rat uterus the enzymes for the incorporation of serine, glycine and formate into adenine and guanine, and those responsible for incorporation of some seven amino acids into proteins are increased many fold after in vivo administration of oestradiol-17β, referred to hereafter as oestradiol. It is noted that oestradiol has

little effect either on the synthesis of the pyrimidine bases of nucleic acids, or on the stimulation of the activation of thirteen other amino acids tested, thus indicating a certain degree of specificity of the hormone action. In addition to these effects Emmelot & Bosch (1954) have shown that oestrogen pretreatment increases the rate of biosynthesis of cholesterol in the mouse uterus.

Thus the rates of biosynthesis of a number of important constituents of the uterine cell are increased by oestrogen. In reviewing his results, Mueller (1957) suggests that the oestrogen increases the rates of many enzyme systems by activating preformed enzyme molecules, and by increasing the rate at which enzyme molecules are produced de novo.

Mueller also found that oestradiol added to enzyme systems in vitro is without effect. However, irregular increases in the enzymic rates were obtained with 4-hydroxy- and 2-hydroxyoestradiol, suggesting that they may be the 'active' forms of oestrogen (Mueller, 1955). The fact that there is no oestrogen effect in vitro suggests that oestrogen may not have a primary role in these systems. The goal in studies of mechanism of action is the development of in vitro systems, preferably derived from 'target' organs, in which reproducible and measurable changes can be elicited

by biologically active substances in physiological concentration.

With this point in mind, it is not surprising that much attention has recently been paid to a second approach, the demonstration of an in vitro effect, by Villee, Talalay and their co-workers. Hagerman & Villee (1952, 1953) and Villee & Hagerman (1953) showed that the addition of oestradiol to human endometrium or placenta slices in vitro produced an increase in the rate of utilisation of oxygen, pyruvate and acetate. Upon further examination of this effect with a soluble preparation from human placenta, Gordon & Villee (1955) and Villee & Gordon (1955) showed a stimulatory effect of oestrogenic hormones upon the reduction of DPN* (but not of TPN) in the presence of isocitrate. In order to explain these observations it was suggested that the hormone combines with an inactive form of a DPN-linked isocitric dehydrogenase, thereby rendering it enzymatically active. A kinetic analysis (Gordon & Villee, 1956) provides the basis for the enzyme activation concept.

The observations of Villee and associates have been confirmed and extended by Talalay & Williams-Ashman (1958) who found that in more highly purified preparations of human placenta, the oestrogen effects disappeared but could be

* DPN, DPNH, TPN and TPNH represent oxidised and reduced di- and triphosphopyridine nucleotides respectively.

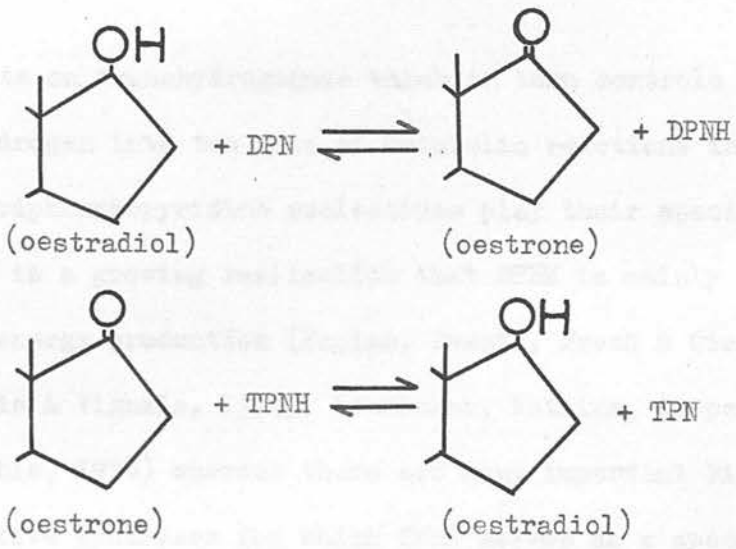
restored by the addition of catalytic amounts of TPN. They concluded that the effect of the hormone is not upon isocitric dehydrogenase (reaction c) but upon a transhydrogenase (reaction b) as shown in the following coupled reactions:-

(a) isocitrate + TPN $\rightleftharpoons$ α-oxoglutarate+CO_2+TPNH

(b) TPNH + DPN $\rightleftharpoons$ DPNH + TPN

(c) isocitrate + DPN $\rightleftharpoons$ α-oxoglutarate+CO_2+DPNH

Thus the locus of action of oestrogen was shifted from isocitrate to transhydrogenase system. The similarities in properties and kinetics of the transhydrogenase and the oestradiol dehydrogenase of human placenta described by Langer & Engel (1958) led Talalay & Williams-Ashman to conclude that the two enzymes are identical. This suggests that the reversible oxidation and reduction of the steroids themselves constitutes a steroid-activated transhydrogenase system according to the equations:-



Sum: DPN + TPNH $\rightleftharpoons$ DPNH + TPN

This concept would place the steroid hormones, including, in theory, many adreno-corticœsteroids and androgens as well as oestrogens which show the reversible change

$$>CO \rightleftharpoons >CHOH,$$

in the position of coenzymes similar to some vitamins of the B complex group. However, Villee, Hagerman & Joel (1960) in their latest review produce evidence against the coenzyme theory including the fact that transhydrogenase and oestradiol dehydrogenases activities can be separated by electrophoresis.

Whether the oestrogen 'activates' transhydrogenase as visualised by Villee, or functions as a coenzyme as suggested by Talalay, it is agreed that the hormone exerts

effects on transhydrogenase which in turn controls the flow of hydrogen into two sets of metabolic reactions in which di- and triphosphopyridine nucleotides play their specific roles. There is a growing realisation that DPNH is mainly concerned with energy production (Kaplan, Swartz, Frech & Ciotti, 1956; Vignais & Vignais, 1957; Lehninger, Watkins, Cooper, Devlin & Gamble, 1958) whereas there are many important biological reductive syntheses for which TPNH serves as a specific hydrogen donor. These include the biosynthesis of fatty acids and steroids, hydroxylations, reductive carboxylations, and the synthesis of ascorbic acid (Horecker & Hiatt, 1958; Dickens, Glock & McLean, 1959; Klingenberg & Bücher, 1960). Therefore the action of the pyridine nucleotide transhydrogenase could facilitate the capture of energy from the oxidation of TPNH via DPNH, and could also divert hydrogen flow so as to provide TPNH and thus stimulate anabolic processes.

The present position appears to be that a locus of action of oestrogen is upon a transhydrogenase, but several other factors must also be kept in mind. First we have as yet no information concerning the quantitative importance of the oestrogen-activated transhydrogenase in the functioning of the cell. Secondly, the phenomenon has been investigated most thoroughly in the human placenta, a

tissue far from being an ideal 'model' for studying the mechanism of action of oestrogen.

With these points in mind, an account is presented in SECTION II of this thesis of the attempt to demonstrate the oestrogen-activated transhydrogenase in the 'target' organs known to show peculiar sensitivity to oestrogen, including the rat uterus and the chicken oviduct.

An attempt has also been made to verify Talalay's "coenzyme theory" by studying the effect of adrenocortical steroid hormones on transhydrogenase activity in tissues with hydroxysteroid dehydrogenase activity. The steroid hormones possess groups which can undergo the change

$$>C = O \rightleftharpoons >CHOH$$

and some of the hydroxysteroid dehydrogenases which catalyse these reactions have dual pyridine nucleotide specificity so that transhydrogenase action might proceed as follows:

$$DPNH + >CO \rightleftharpoons DPN + >CHOH$$

$$TPN + >CHOH \rightleftharpoons >CO + TPNH$$

$$\text{sum:-} \quad DPNH + TPN \rightleftharpoons DPN + TPNH$$

In addition to the investigation of the <u>in vitro</u> effect of oestrogens on transhydrogenation, an attempt was made to study the effect of these substances on

transhydrogenation *in vivo* by measuring changes in the levels of oxidised and reduced DPN and TPN in the rat uterus after ovariectomy and subsequently on administration of oestrogen. A knowledge of the effects of oestrogenic hormones administered *in vivo* on the DPN/DPNH and TPN/TPNH ratios in the rat uterus might give some indication of the possible physiological significance of the transhydrogenation theory.

There is evidence that concentrations of pyridine nucleotides in tissues are influenced by hormones. Thus McLean (1958) reported increases in DPN and TPNH but no change in DPNH or TPN concentrations in the lactating mammary glands of rats. The liver and diaphragm of rats given alloxan, growth hormone, thyroxine and thiouracil show changes in DPN and DPNH but little changes in TPN and TPNH (Glock & McLean, 1955d). Greenberg & Glick (1960) reported increases in concentrations of both oxidised and reduced forms of DPN and TPN in the adrenal glands of rats after the administration of adrenocorticotrophin (ACTH). Recently Loring, Spencer & Villee (1961) showed that there is no effect on the DPN/DPNH ratio in the liver, kidney and muscle of castrate male rats after administration of testosterone, but the TPN/TPNH ratio is increased in hormone treated animals.

The results of determinations of DPN, DPNH,

TPN and TPNH concentrations in the rat uterus after ovariectomy and after oestradiol administration are presented in SECTION III of this thesis.

In addition to the determination of pyridine nucleotides, the quantitative activities of DPN- and TPN-dependent dehydrogenases in the rat uterus with respect to oestrogenic hormonal status were investigated. In particular, the TPN-dependent dehydrogenases of the hexose monophosphate ('shunt') pathway of glucose oxidation might be influenced by oestrogen, since increased activity of the 'shunt' would help to provide adequate supplies of both TPNH, required in many biosyntheses processes (Horecker & Hiatt, 1958; Dickens et al, 1959) and of ribosephosphate necessary in the biosynthesis of nucleotides and nucleic acid (see Glock & McLean, 1958).

Oestrogen pretreatment of spayed rodents is known to increase the levels of many enzymes in the uteri e.g. β-glucuronidase (Fishman & Farmelant, 1953), alkali phosphatase (Harris & Cohen, 1951), peroxidase (Lucas, Neufeld, Utterback, Martin & Stotz, 1955) and lactic dehydrogenase-DPNH oxidase (Bever, Velardo & Hisaw, 1956). There is evidence that hexose monophosphate 'shunt' enzymes are subjected to hormonal control. Thus Glock & McLean

(1954) reported that glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) activities increase in the mammary glands of the rat during lactation. The same authors (Glock & McLean, 1955c) found a decrease in 6PGDH activity in the liver of alloxan-diabetic rats but both G6PDH and 6PGDH activities rise after insulin administration. An increase of the same enzymes in the liver of rats treated with thyroxine was reported by Glock, McLean & Whitehead (1956b). Huggins & Yao (1959) find an increase in hepatic 6PGDH activity of gonadectomised rats of both sexes after 13 days oestrogen administration, but the activity of G6PDH is unchanged under the same conditions.

The effects of <u>in vivo</u> administered oestrogen on another important TPN-dependent enzyme, isocitric dehydrogenase (ICDH) and on the DPN-dependent enzyme, lactic dehydrogenase (LDH), have also been studied for comparison so that it will be possible to say whether there is any specific oestrogen effect on the dehydrogenases of the hexose phosphate 'shunt'. Details of the investigation are presented in SECTION IV of this thesis.

The finding that there is a selective increase in the activities of 'shunt' enzymes, but not of other TPN-dependent enzymes studied, prompted a study of the effect of

oestrogen on the hexosemonophosphate pathway of glucose oxidation. Although this pathway is of small significance in many tissues (e.g. muscle) compared with the glycolytic pathway, it has been found to be responsible for a significant percentage of glucose oxidation in the liver (see Dickens et al, 1958). It is noted that this pathway is especially active in tissues where rapid biosyntheses are taking place; examples are the lactating mammary gland where milk production occurs at a high rate (Abraham, Hirsh & Chaikoff, 1954; Glock, McLean & Whitehead, 1956a) and the adrenal cortex where active steroid biosynthesis is taking place (Kelly, Nielson, Johnson & Vestling, 1955; Field, Pastan, Herring & Johnson, 1960). It is therefore plausible that the shunt pathway might be of importance in the rat uterus growing rapidly in response to oestrogen.

An additional reason for studying the effect of oestrogen on the hexose monophosphate shunt is the report of Roberts & Szego (1953) that oestrogen administered to the castrate rat, caused an increased glucose utilisation accompanied by lactate accumulation. The lactate accumulation however decreases markedly by the 20th hour after oestrogen administration while glucose utilisation is still increasing. This might well be interpreted as the take-over from glycolysis by the 'shunt' pathway of

glucose oxidation after the 20th hour.

Accordingly the comparative significance of both pathways of glucose oxidation in the uterus of castrated and oestrogen treated rats was investigated and is reported in SECTION V of this thesis.

A general discussion is found in SECTION VI.

SECTION II

ATTEMPTS TO DEMONSTRATE THE PRESENCE OF A STEROID-ACTIVATED PYRIDINE NUCLEOTIDE TRANSHYDROGENASE IN TISSUES OF ANIMALS AND HUMAN SUBJECTS.

INTRODUCTION

Pyridine nucleotide transhydrogenase is the enzyme which catalyses the transfer of hydrogen between pyridine nucleotides as in the equation:-

$$DPN + TPNH \rightleftharpoons DPNH + TPN$$

The existence of this enzyme was foreshadowed by Ball (1942) who suggested that a flavoprotein might carry out this function, but it was Colowick, Kaplan, Neufeld & Ciotti (1952) who first provided the evidence for pyridine nucleotide transhydrogenase in Pseudomonas fluorescens. Since then this enzyme has been demonstrated in the mitochondria of many animal tissues (Kaplan, Swartz, Frech & Ciotti, 1956; Navazio, Ernster & Ernster, 1957; Vignais & Vignais, 1957; Humphrey, 1957). Humphrey (1957) reported that transhydrogenase occurs in the particulate fraction of cells of animal tissues especially heart muscle, and to a small extent in skeletal muscle, kidney and liver. Negligible activity was found in the brain, prostate, seminal vesicles, spleen and testis. More recently Grant & Mongkolkul (1959) found only negligible transhydrogenation in adrenal mitochondria.

A distinguishing feature of the animal mitochondrial transhydrogenases is that they are not activated by the addition in vitro of certain oestrogens (Villee, 1955).

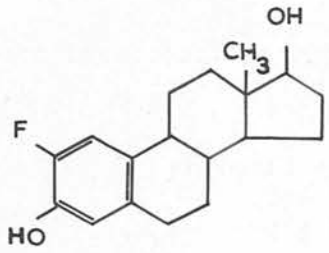
They differ thus from a second group of pyridine nucleotide transhydrogenases which are present in the cell sap (Villee, 1955; Stein & Kaplan, 1959).

These soluble oestrogen-sensitive pyridine nucleotide transhydrogenases, first found in human placenta, have already been referred to in the General Introduction (p. 4-8). In addition Hollander, Jona & Smith (1958) were able to demonstrate oestrogen stimulated transhydrogenation in the soluble fraction of human breast tissue. However more than half of the specimens tested lacked the ability to respond to oestrogen. It was hoped that this might offer a diagnostic test for those breast tumours which were hormone dependent, but examination of an extended series of cases failed to give consistent results (Hollander, 1958). Hurlock & Talalay (1958a & b) reported that 3α-hydroxy and 3-oxosteroids are able to stimulate transhydrogenation in purified preparations of rat liver. Thus prior to the start of this investigation only human placenta and human breast tissue were shown to contain the oestrogen-activated transhydrogenases. Nevertheless oestrogen added *in vitro* has long been known to increase the rate of oxygen utilisation by many 'target' tissues. It is often quoted (Villee, 1959; Villee *et al*, 1960) that the increases in O_2 consumption in these tissues are due to the activation of transhydrogenase

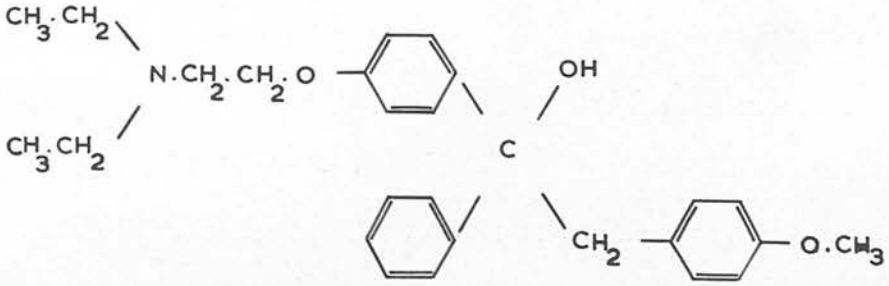
by oestrogen. In no case, however, has this been shown definitely to be due to transhydrogenase activity. Hence a closer examination of these tissue from 'target' organs is desirable.

PLAN OF INVESTIGATIONS

1. The first line of investigation was an attempt to demonstrate the presence and quantitative significance of steroid-activated pyridine nucleotide transhydrogenase in some oestrogen 'target' organs. Preliminary experiments were carried out with human placenta as a model tissue for testing the practicability of the steroid-activated transhydrogenase assay, and for investigating the effect of certain substances on the enzyme. The substances used were 2-fluoro-oestradiol-17β, MER-25 and amphenone-B (formulae p.p. 18). The strength of the C-F bond is known to be such that no replacement, in enzymatic systems, of the F by a hydroxyl group is possible in the first compound mentioned. An investigation of the effect of 2-fluoro-oestradiol-17β on transhydrogenase in the human placenta is of interest in view of the postulation by Mueller (1955, see p.3) that hydroxylation of oestradiol at C-2 or C-4 may be necessary before the compound is biologically active. MER-25 was found by Learner, Holthaus & Thompson (1958) to antagonise the uterine and vaginal responses to oestrogen in competitive fashion, and amphenone-B is a non-steroidal substance with a progesterone-like action in inhibiting the response to oestrogen in the growth of hen oviduct (Hertz et al, 1955). It is therefore

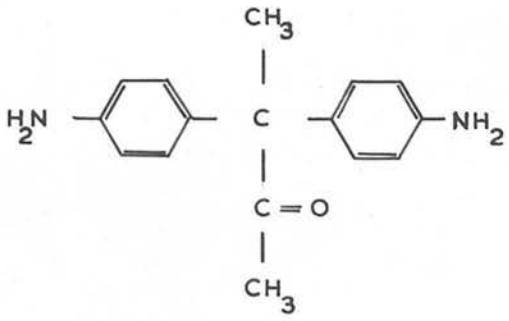


2-fluoro-oestradiol-17β



(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-(p-methoxyphenyl) ethanol

(MER-25)



3,3-Bis[p-aminophenyl]-2-butanone

(Amphenone-B)

interesting to know whether these oestrogen antagonists have any effect on the oestrogen-stimulated transhydrogenase system in the human placenta.

In general, tissues were chosen for study according to the following criteria:-

(a) Those which respond extremely well by growth or marked change in cell morphology to oestrogen administered in vivo.

(b) Those which show an increase in oxygen consumption, when oestrogen is added in vitro, but in which it has not been shown conclusively that the effect is due to the stimulation of transhydrogenase.

Most of the tissues studied belong to these categories.

(c) Placentae from a number of animals were also examined for oestrogen-sensitive transhydrogenase activity in order to find if the phenomenon in this tissue occurs widely in mammalian species, or if it is restricted to man. This possibility is paralleled by the fact that oestriol appears to be characteristic of man, there being no satisfactory evidence for the occurrence of this steroid in other species (see Merrill, 1958).

Oestradiol-17α, isolated from the urine of the pregnant mare (Hirschmann & Wintersteiner, 1937) and from

rabbit urine after oestrone administration (Stroud, 1939) has not so far been found in man. There would therefore appear to be a qualitative species difference in the way in which oestrone is reduced (Marrian, 1958). If transhydrogenase is a key enzyme in the mechanism of oestrogen action it was considered to be of interest to look for this enzyme in rabbit placenta and to test the effect of oestradiol-17α on it. Oestradiol-17α is without effect on the enzyme from human placenta (Gordon & Villee, 1956).

The tissues examined were the following:

Human endometrium: This is known to undergo rapid growth after oestradiol administration (Hagerman & Villee, 1953) and an increase in oxygen consumption has been shown to occur when oestradiol is added *in vitro* (Villee & Hagerman, 1952).

Rat and guinea-pig uterus: It is well known that the uteri of small rodents undergo rapid growth in response to oestrogenic steroids administered *in vivo* (Bulbring & Burn, 1935; see also Fig. I. 1). Khayyal & Scott (1931) also reported an increase in oxygen consumption by the uteri of rats or mice on addition of oestrogens *in vitro*. It would thus appear that if transhydrogenase plays any significant role in the mechanism of action of oestrogens it should be present in the uterus and its activity should be influenced

by oestradiol.

Rat vagina: The changes in cell type in this tissue in response to administered oestrogen (Stockard & Papanicolaou, 1917) form the basis of the well known vaginal smear method for the bioassay of oestrogens and justify the choice of this tissue for investigation of transhydrogenase activity.

Chicken oviduct: This organ shows a dramatic growth response to oestradiol administered *in vivo*. Some aspects of this growth response have been studied by Hertz, Tullner, Schricker, Dhyse & Hallma (1955).

Ox pituitary gland was chosen for study since the anterior lobe secretes gonadotrophin which increases oestrogen production, and oestrogen in turn influences the secretion of the anterior lobe (see Burrows, 1949b). Furthermore Meyer (1956) has shown that oestrogen may cause the development of pituitary tumours. An increase in oxygen consumption by pituitary tissue on adding steroid oestrogens *in vitro* has been demonstrated for the rat by Victor & Anderson (1938) and for man by Gaull & Villee (1959).

Other tissues: In 1957 Humphrey reported weak transhydrogenase activity in the soluble fraction of rat spleen and heart cells but did not consider possible effects of steroids. This has now been investigated.

2. The second line of the investigation was to clarify the postulation that "...all (hydroxysteroid dehydrogenases with dual pyridine nucleotide specificity) function as transhydrogenases..." (Talalay & Williams-Ashman, 1958). The apparent identity of the placental transhydrogenase with oestradiol dehydrogenase, and of a rat liver transhydrogenase with 3α-hydroxysteroid dehydrogenase (Hurlock & Talalay, 1958a and b) may be quoted as examples. Hence it appeared to be interesting to find if other steroids such as those with a hydroxy group at C-11 stimulate the transfer of hydrogen between pyridine nucleotides in the adrenal and liver where the corresponding steroid-dehydrogenases have been demonstrated (Amelung, Hubener, Roka & Meyerheim, 1953; Fish, Hayano & Pincus, 1953). Similarly it appeared to be of interest to find if the 3β-hydroxysteroid dehydrogenase, present in the microsomal fraction of adreno-cortical cells (Pincus, 1958) shows any transhydrogenase activity.

3. The last investigation of this Section was undertaken after the report by Scott & Lisi (1960), that significant transhydrogenase activity exists in the so called "supernatant" fraction of the rat adrenal cells. The authors did not mention the possible stimulation of this

enzyme by steroids. This observation was reinvestigated since it appears to conflict with results already obtained here, and could possibly be explained by contamination of the supernatant fraction of Scott & Lisi with mitochondria. It was not originally intended to study the mitochondrial transhydrogenase, since it is not stimulated by steroids added *in vitro*, and is, therefore, unlikely to have a primary role in the mechanism of action of steroid hormones.

EXPERIMENTAL

Materials

Term placentae from women were obtained within an hour of delivery. Uteri were those removed surgically in treatment of carcinoma of the cervix, and adrenal glands were those removed surgically from patients with advanced carcinoma of the breast. Placentae of rats, guinea-pigs and rabbits were removed surgically in late pregnancy since these animals usually eat the placenta after the birth of the young. The placentae, adrenal glands and pituitaries of pigs and oxen were obtained as soon as possible after the slaughter of the animals. Chicken oviducts were obtained fresh from the Poultry Research Centre, Edinburgh.

All tissues were transported to the laboratory packed in crushed ice and experiments were made without delay.

Preparation of tissue homogenates

All reagent solutions and apparatus used were cooled in crushed ice and all operations were done at about 0°.

Placenta: Small pieces of tissue of about 1 cubic centimeter volume were cut from the organ and washed thoroughly with physiological saline to remove blood.

Connective tissue was avoided as far as possible. The tissue was then homogenised for 1 min. with 4-5 volumes of 0.25 M sucrose in a Nelco Blender (Measuring & Scientific Equipment Ltd. (M.S.E.) London), operated at 60 volts to reduce the speed and hence damage to cell components.

Human endometrium: The uterus obtained was cut in half longitudinally and the inner endometrium layer scraped off and used in making a 20% homogenate. This tissue was homogenised in a cone-shaped ground glass homogeniser for 1 min. in 0.25 M sucrose solution. This method of homogenisation was used with all the following tissues.

Rat and guinea-pig uterus: The uteri from adult 6-8 month old animals, killed by decapitation, were separated from adhering fat and connective tissues and slit open longitudinally. From three to six uteri were pooled together before homogenisation. Uteri from rats ovariectomised 2 weeks earlier were used in one experiment.

Rat vagina: Usually when uteri of rats were removed, the vaginae were also kept. They were trimmed free from fat and connective tissues. About 6 vaginae were used in each experiment.

Chicken oviduct: Four young chicks ($5\frac{1}{2}$ weeks old) were used in each experiment. Three birds were kept as a control, and the other was injected subcutaneously with 10 µg

oestradiol benzoate every second day over a period of 12 days. Birds were then killed by breaking their necks, oviducts were removed and homogenised as already described.

Ox pituitary glands: The glands were dissected from tough connective tissue capsules and divided into anterior and posterior lobes. Each part was homogenised separately.

Human adrenal gland: The glands were freed from adhering blood and fat, and connective tissue was removed as far as possible. The whole cortical fraction was used without attempting further division into the different zones. The two glands investigated were removed from the same patient in a two stage, bilateral adrenalectomy. The first gland weighed 5 g. The second, removed a week later after the patient had received 80 units ACTH intramuscularly daily for four days before operation, weighed 8 g.

Ox adrenal gland: The gland was cleaned as described for the human gland. The medulla was discarded, only the cortex was investigated.

Rat adrenal gland: Whole glands were used first, and in one experiment the cortex was dissected away and only the medulla tissue was homogenised.

Tissue fractionation

The preparation of cell fractions was based on

the method of Hogeboom (1955) slightly modified. The homogenised tissue was centrifuged for 10 min. at 700 g. to remove unbroken cells and nuclei. As a result about 10% of the mitochondria released from the cells are sedimented with this fraction but as it was not desired to have a quantitative yield of mitochondria this is not important.

The mitochondria were sedimented by centrifugation at 5,000 g. for 20 min. Normally they were washed once by resuspending in sucrose solution and homogenising for 1 min. using a round glass-bulb homogeniser in a well fitting plastic tube and resedimenting at 5,000 g. for 20 min.

The microsomes were separated from the supernatant fluid from the mitochondrial preparation by centrifuging either for 30 min. at 105,000 g. in a Preparative Spinco Model L Ultracentrifuge, or for 2 hr. at 20,000 g. in an M.S.E. Refrigerated Centrifuge with high speed head attachment.

When only the soluble enzymes of the cell sap were required the homogenate was centrifuged once only at the speed used for separating microsomes.

The crude cell fractions were usually assayed without further purification, but in some cases dialysis or ammonium sulphate fractionations of soluble enzymes were

carried out. Enzymic activities were normally determined within one day, otherwise the enzyme preparations were stored overnight in a deep freeze (-15°). This storage results in insignificant loss of activity of the crude transhydrogenase preparation from human placenta.

Dialysis

When dialysis was required, the enzyme preparations were put in a cellophane bag and dialysed against excess 0.25 M sucrose solution for 18 hr. in the refrigerator at about 4°. The sucrose solution was changed at least once during the process. Small amounts of precipitates of denatured proteins appearing during dialysis were centrifuged down and discarded. Only the clear supernatants were used in the enzymic assays.

Ammonium sulphate fractionation

In some experiments fractionation of the enzymic preparations was carried out at 0° with ammonium sulphate. The precipitates which formed upon the addition of calculated amounts of the salt were collected by centrifugation and were dissolved in 0.01 M tris buffer pH 7.4.

Nitrogen determination

The nitrogen content of each enzyme preparation was routinely determined as a means of comparing different preparations. The nitrogen contents of supernatant fractions are also a good indication of the completeness of the homogenisations. For the determination, samples dialysed to remove sucrose were digested with sulphuric acid containing a mercury catalyst, followed by direct Nesslerisation (Le Page, 1957).

Enzymic determinations

Two methods had been employed in the determination of pyridine nucleotide transhydrogenase.

(a) Spectrophotometric method.

This method was based on that of Gordon & Villee (1956). Full details of the reactants are shown under Fig. II. 1. Briefly, the incubation mixture contains excess DPN and a TPN reducing system, having substrate (isocitrate or glucose-6-phosphate) and corresponding dehydrogenase present in excess. Only catalytic amounts of TPN are present. The only limiting step is thus the transfer of hydrogen from TPNH to DPN. This is measured as an index of transhydrogenase activity. The formation of reduced DPN is followed by measuring the increase in

optical density at 340 mμ, using the Unicam SP500 spectrophotometer. A special copper container with 4 compartments, designed to take the 1 cm. silica cuvettes used, was made so that the cuvettes could be maintained at desired temperatures in a thermostat and removed for short periods in order to measure optical densities. It was found that incubation at 37° increased the activity of human placental transhydrogenase by two fold over the value found at room temperature (20°). However, room temperature incubations were employed for heat labile preparations such as those of the rat uterus. Otherwise the precipitation of protein interfered with the optical measurements.

After the incubations, alcohol dehydrogenase and acetaldehyde were usually added to the mixture to test if the increase in the optical density was in fact due to DPNH production. Alternatively when no increase in optical density was observed alcohol dehydrogenase plus ethanol were added to the system to check that DPN was still present in appreciable quantity and that the negative results were not caused by destruction of this coenzyme.

(b) α-oxoglutarate determination.

This method for transhydrogenase assay was not used as extensively as the spectrophotometric method. The reason for introducing it was to confirm the results obtained

spectrophotometrically, especially in cases where the enzyme preparations were heat labile and became turbid after long incubations thus preventing accurate optical measurement. The reaction mixture is the same as for the spectrophotometric method (full details under Fig. II. 1), but isocitrate only is used in generating TPNH. The rate of formation of α-oxoglutarate from isocitrate, which is measured, depends on the rate of the reoxidation of TPNH to TPN in which the activity of transhydrogenase is the governing factor. α-oxoglutarate is determined by the method of Friedemann & Haugen (1943) as used by Loring & Villee (1957).

The addition of steroids

Crystalline steroids were initially dissolved either in propylene glycol or dioxane. Both solvents were found to be equally suitable for use with the human placental enzyme system. It was found, however, that propylene glycol could be reduced by a rat liver soluble enzyme requiring DPN as coenzyme and thus interfered with the optical method of assaying transhydrogenase. Although many other tissues, including human placenta, are devoid of such a "propyleneglycol dehydrogenase," dioxane, which is not metabolised in this way, was used as a solvent for

steroids in all subsequent investigations.

RESULTS

(a) Experiments on human placenta

In preliminary experiments it was possible to confirm that oestradiol and oestrone greatly stimulate the transhydrogenase activity, oestriol has only a very slight effect while oestradiol-17α has none. The result of a typical spectrophotometric assay of transhydrogenase in a human placental preparation is shown in Fig. II. 1, and that of an α-oxoglutarate determination is shown in Fig. II. 6.

The fraction of the soluble placental enzyme preparation precipitated by 40% saturation with ammonium sulphate also showed oestrogen-sensitive transhydrogenase activity (Fig. II. 2) and was found to require minute amounts of TPN as described by Talalay & Williams-Ashman (1958). (The crude preparations do not require added TPN). The presence of DPN- and TPN-linked oestradiol dehydrogenase was also demonstrated in the purified fraction (Fig. II. 3).

As an extension of the study of the human placental transhydrogenase reported by others, the effects on the enzyme of certain less common steroids and of oestrogen antagonists were studied. Fig. II. 4 shows that 2-fluoro-oestradiol-17β is as effective as oestradiol

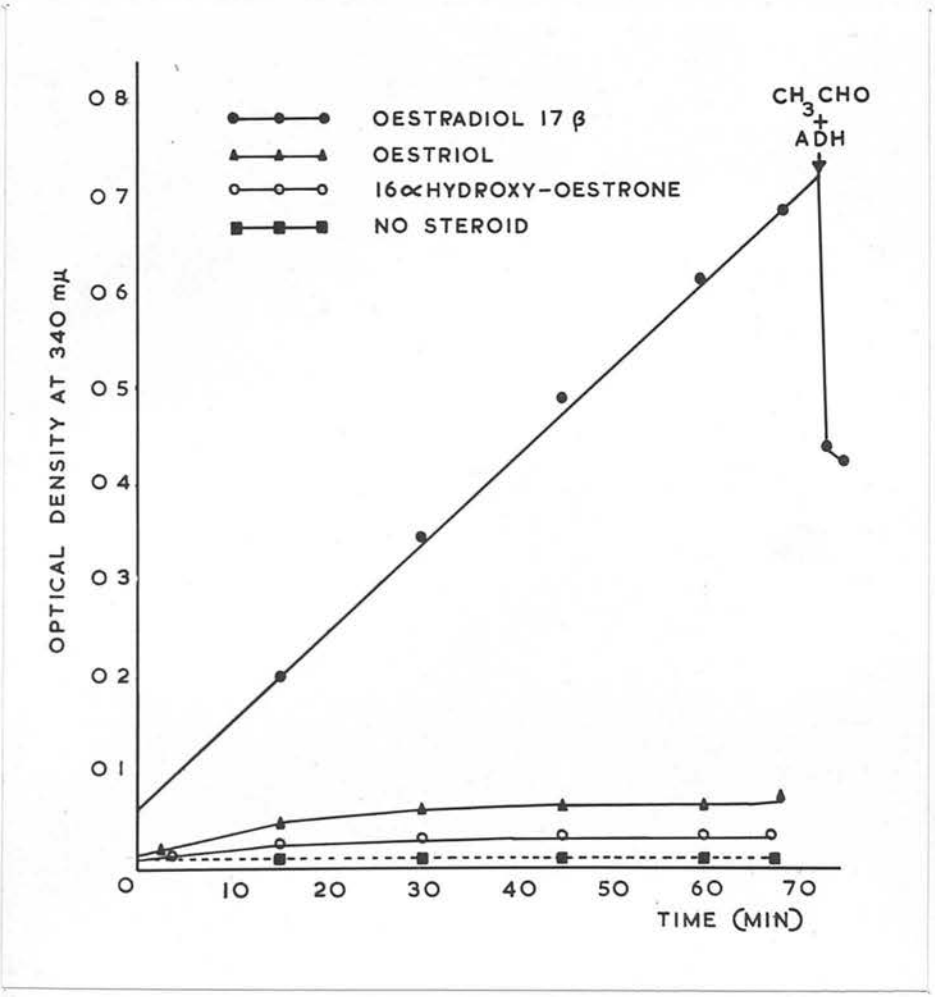


Fig. II. 1. Effects of oestradiol, oestriol and 16α-hydroxyoestrone on transhydrogenase activity in the crude human placental preparation. The 3 ml final volume incubation mixture contained 100 mM of tris buffer, pH 7.4; 3 mM of $MnCl_2$; 2 mM of DL-isocitrate; 0.25 mM of DPN and 1 ml of the enzymic extract (containing 1 mg N_2 per ml). 10 μg of steroid (final concentration 1.3 X 10^{-5}M) was added when indicated. Blank cell was devoid of DPN. The decrease in the optical density when alcohol dehydrogenase and acetaldehyde were added at 70 min. confirmed that the original rise in the absorbance at 340 mμ was due to DPNH produced. (If it is not otherwise stated, the incubation mixture used in the following Figures in Section II contain the same reaction mixture as above).

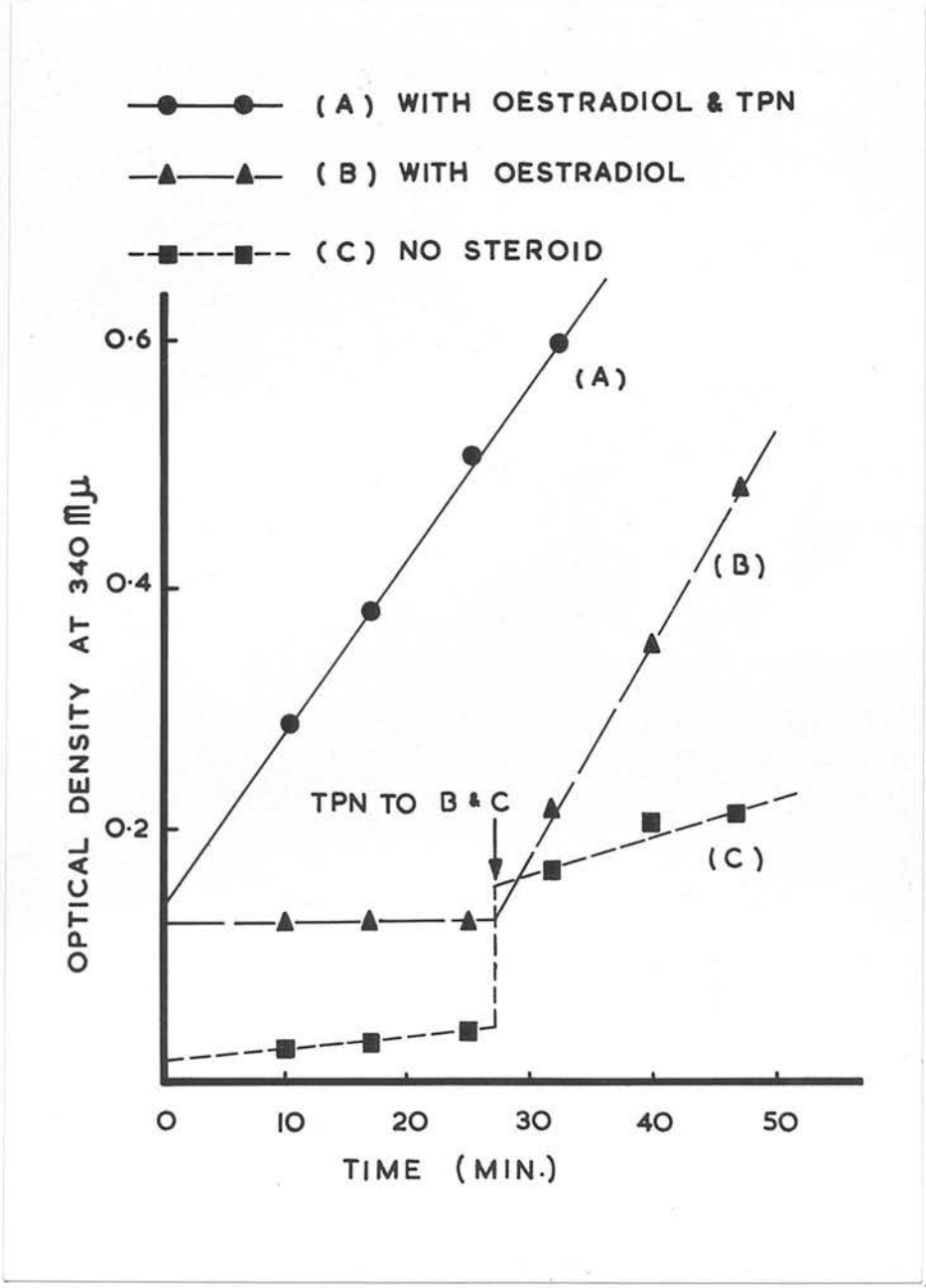


Fig. II. 2. Demonstration of steroid activated transhydrogenase. The enzyme preparation was the fraction of a human placental cell sap preparation precipitated by 40% saturation with ammonium sulphate. Both TPN (4×10^{-6} M) and oestradiol (1.3×10^{-5} M) are seen to be necessary for maximum effect.

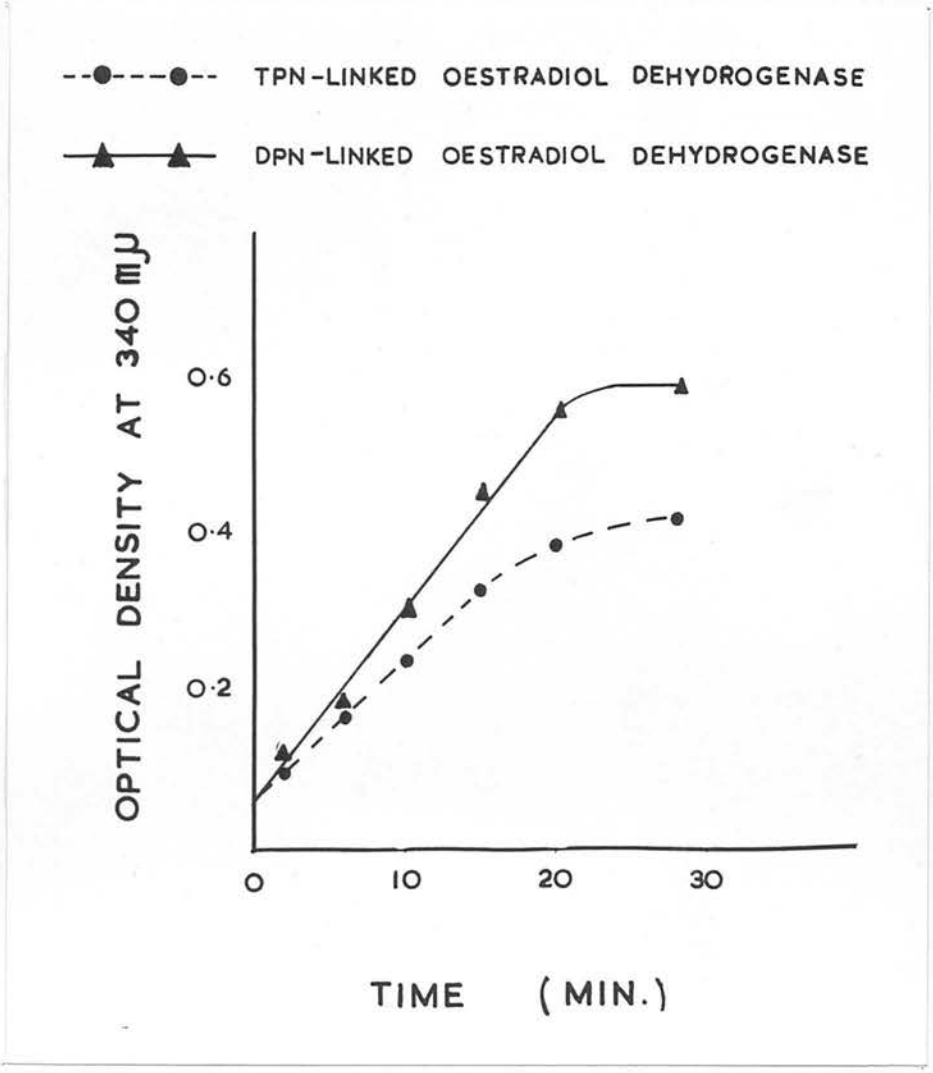


Fig. II. 3. Demonstration of DPN- and TPN-linked oestradiol dehydrogenase in human placenta. The enzyme preparation was the fraction of a human placental cell sap preparation precipitated by 40% saturation with ammonium sulphate. The incubation mixture contained 30 mM $Na_3HP_2O_7$ buffer, (pH 9), 20 mg human serum albumin, 10^{-4} M oestradiol and 0.25 mM DPN or TPN. The blank cell contained all components other than DPN or TPN. Enzyme preparation added contained 0.14 mg N_2. Final volume was 3 ml.

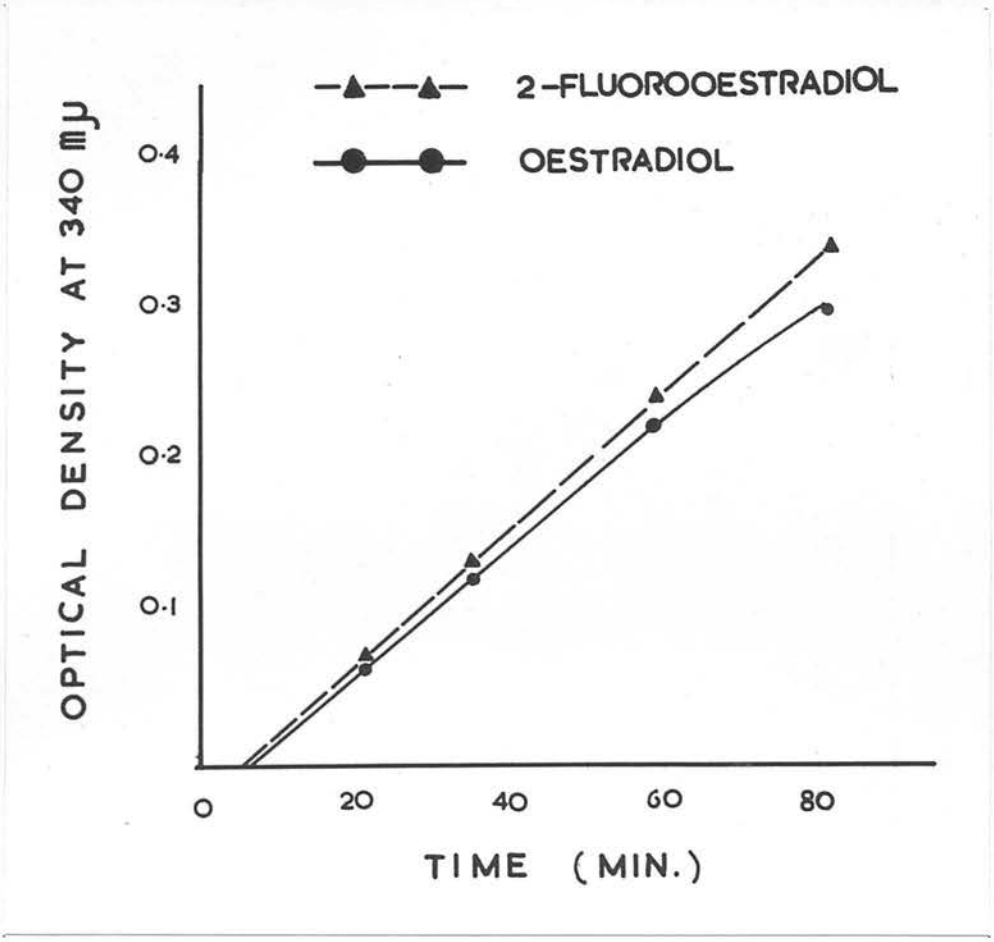


Fig II.4. The effect of oestradiol and 2-fluorooestradiol on human placental transhydrogenase. Reaction mixture as in Fig. II. 1.

in the stimulation of placental transhydrogenase.

MER-25 (in concentrations up to 8×10^{-5}M) and amphenone-B (in concentrations up to 10×10^{-3}M) do not inhibit the transhydrogenase system (Figs. II. 5, II. 6 & II. 7). MER-25 is not readily soluble in water or the incubation mixture and the concentration of 8×10^{-5}M (100 µg per 3 ml.) is almost the maximum which can be obtained.

(b) Attempts to demonstrate steroid activated transhydrogenases in the soluble fraction of cells from human and animal tissues.

From the results summarised in Table II. 1, it may be seen that not all tissues contain demonstrable transhydrogenase activity. Details of the results are as follows:

(i) Tissues which respond sensitively to oestrogen administered in vivo, and/or tissues which give an increase in oxygen consumption when oestrogen is added in vitro.

Human endometrium:

Two experiments have been performed using the endometrium crude soluble fraction. No transhydrogenase activity could be detected by the spectrophotometric technique either in the absence

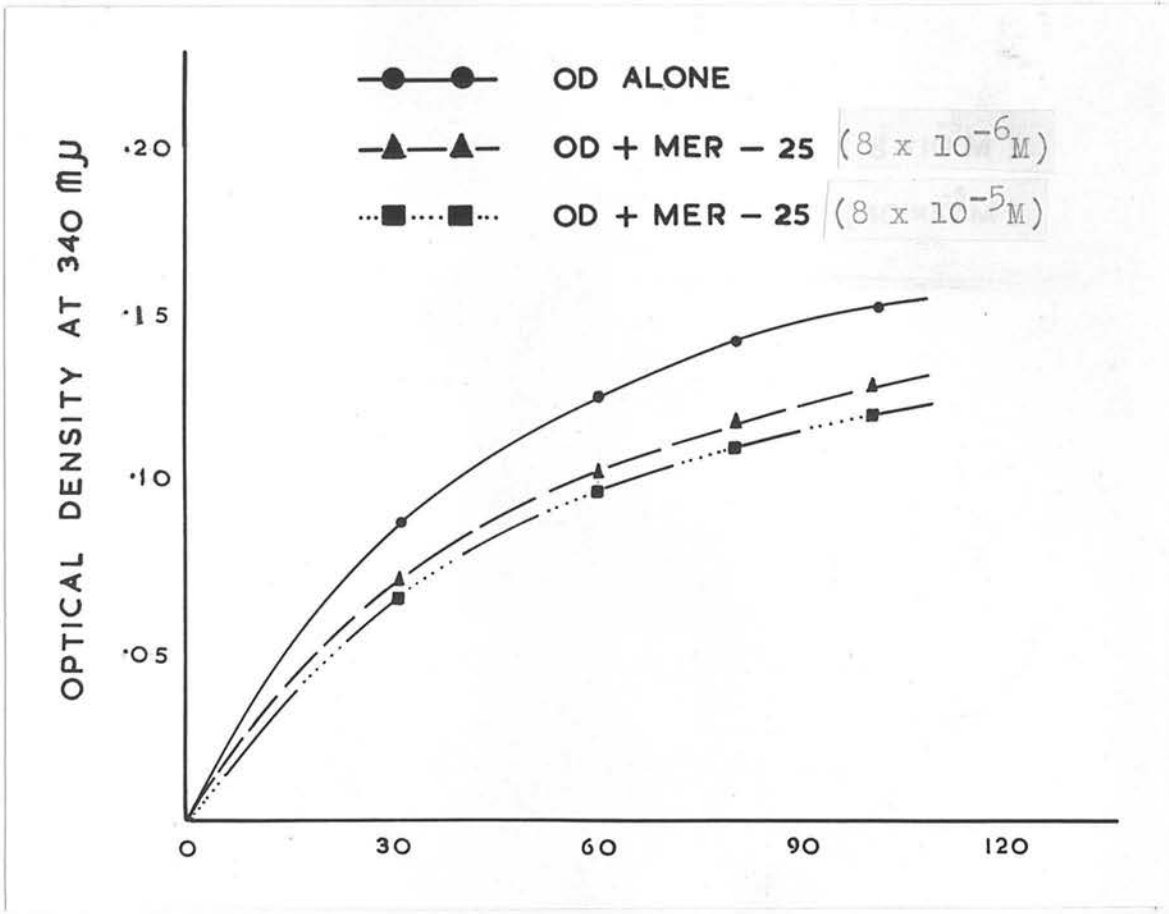


Fig. II. 5. Effect of MER-25 on the oestradiol (OD) stimulated-transhydrogenase of the human placenta.

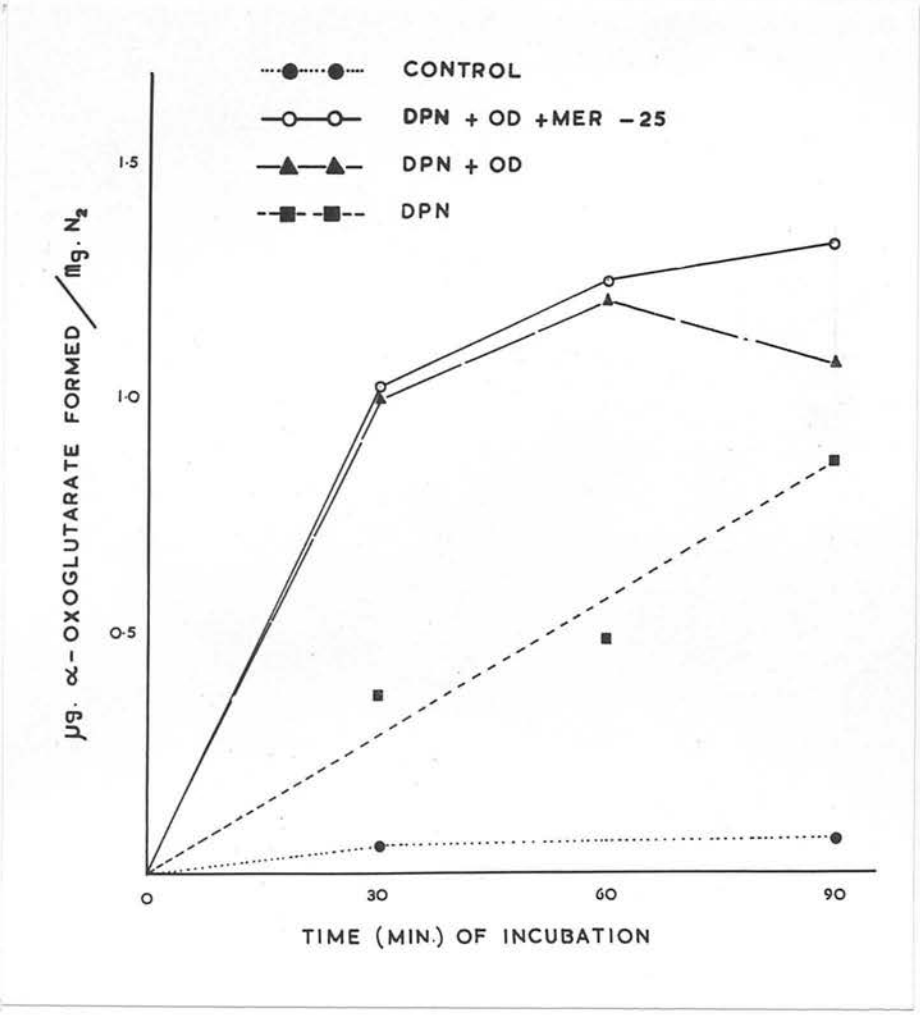


Fig. II. 6. Effect of adding oestradiol (OD) and MER-25 on transhydrogenase activity as measured by α-oxoglutarate method. The reaction mixture contained the same reactants as in Fig. II. 1. MER-25 concentration was 8 X 10^{-5} M.

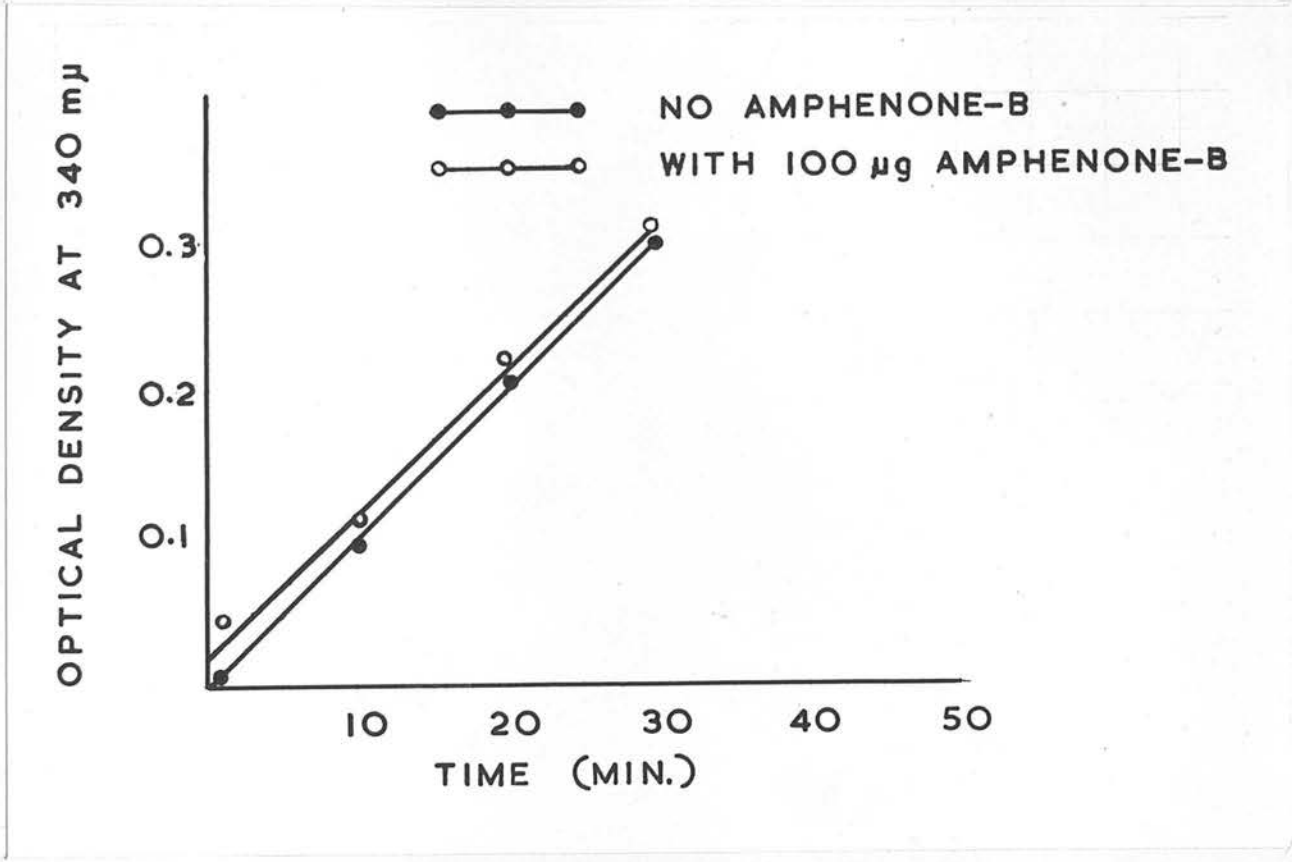


Fig. II. 7. Effect of adding amphenone-B (10^{-4}M) on transhydrogenase activity as measured by the spectrophotometric method.

Table II. 1

Steroid-activated pyridine nucleotide transhydrogenase activity in the soluble fraction of animal cells.

Tissues	Species	No. of expts.	Method of dètn.	Steroid added	Results	Effects of mixing with human placental preparation
Placenta	human	20	Sp*, αOg	oestrone, oestradiol	+ ve	additive effect
				oestradiol-17α, oestriol	- ve	
Endometrium	human	2	Sp.	oestrone, oestradiol	- ve	
Uterus	rat	10	Sp. αOg.	oestrone, oestradiol	- ve	inhibition
Uterus	guinea-pig	1	Sp.	oestradiol	- ve	
Vagina	rat	4	Sp, αOg.	oestradiol, oestriol	- ve	
Oviduct	immature hen	2	Sp.	oestradiol, oestriol	- ve	inhibition
	immature hen + OD**	2	Sp.	oestradiol, oestriol	- ve	
Anterior pituitary	ox	2	Sp.	oestradiol	- ve	
Posterior pituitary	ox	1	Sp.	oestradiol	- ve	
Placenta	rat	1	Sp.	oestradiol	- ve	inhibition
Placenta	guinea-pig	4	Sp, αOg.	oestradiol	- ve	inhibition
Placenta	rabbit	1	Sp.	oestradiol, oestradiol-17α	- ve	inhibition
Placenta	golden-hamster	2	Sp.	oestradiol, oestrone	- ve	
Placenta	pig	1	Sp.	oestradiol	- ve	inhibition
Liver	rat	1	Sp.	oestradiol, cortisol	- ve	
Spleen	rat	1	Sp.	oestradiol	- ve	
Heart	rat	1	Sp.	oestradiol	- ve	
Adrenal cortex	human	2	Sp.	oestradiol, cortisol	- ve	
Adrenal cortex	ox	4	Sp.	oestradiol, cortisol, pregnenolone	- ve	
Adrenal medulla	ox	1	Sp.	oestradiol	- ve	
Adrenal cortex	rat	3	Sp.	oestradiol, cortisol	- ve	
Adrenal medulla	rat	1	Sp.	oestradiol	- ve	

*Sp = Spectrophotometric method
αOg = α-oxoglutarate determination

**OD = oestradiol

or presence of oestradiol, oestrone or oestriol.

Rat and Guinea-pig uterus:

In ten experiments with rat uteri (including one with uteri from ovariectomised rats) and in one with guinea-pig uterus, only negligible activity of transhydrogenase could be detected and this was uninfluenced by oestradiol or oestriol.

The uterine extracts were rather heat labile and appreciable denaturation occurred during incubation at 37°. This interfered with optical measurements but the interference could be decreased by making the measurements at 20°. When the α-oxoglutarate method was used in attempts to detect the activation of transhydrogenase by oestrogen the results obtained were again negative.

These negative results may be due to the presence of an inhibitor in the rat uterine preparation. In order to investigate this the rat uterine preparation was added to an active human placental preparation. Results in Fig. II. 8 show that there is a decrease in transhydrogenation, the extent of which depends on the amount of uterine extract in the mixture. The rat uterine preparation may thus contain an inhibitor of transhydrogenation.

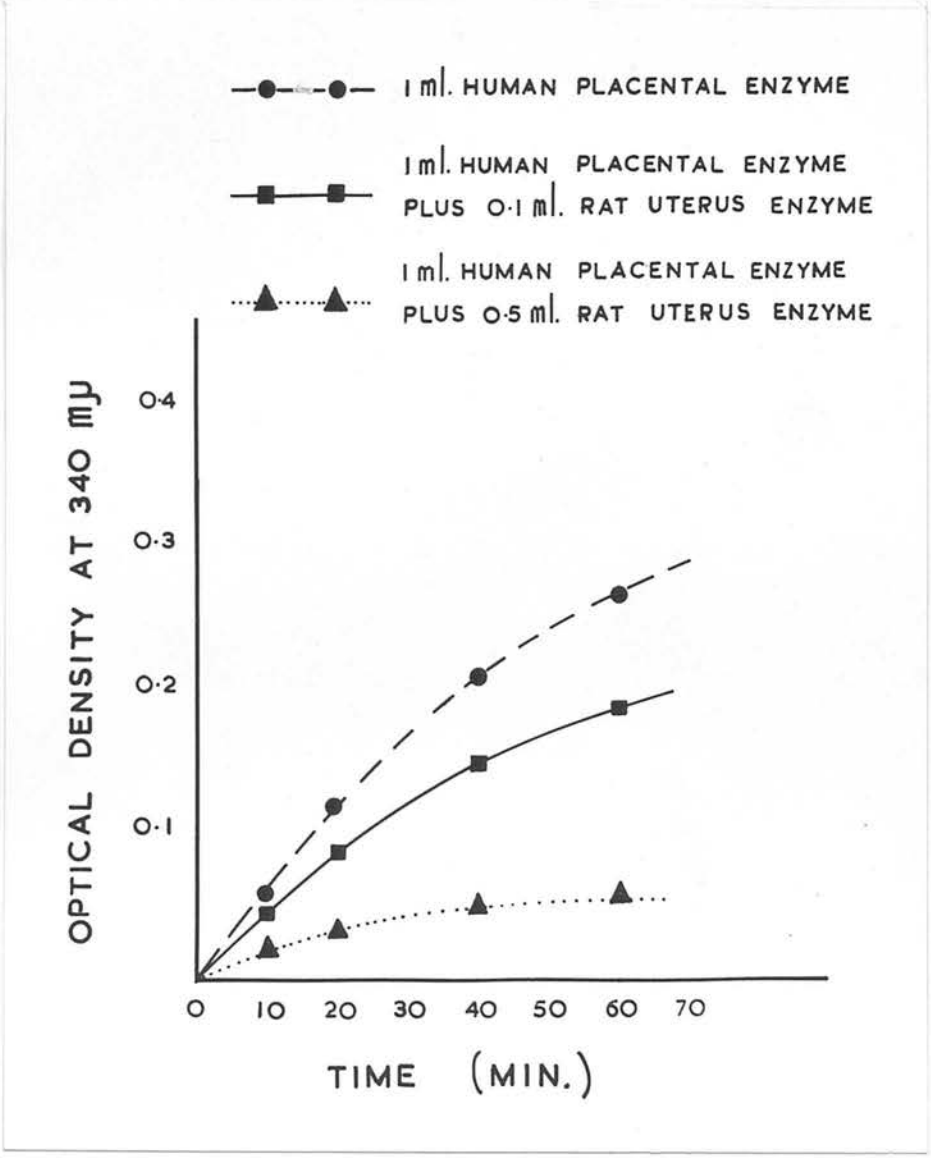


Fig. II. 8. The inhibiting effect on the human placental oestradiol-activated transhydrogenase when different amounts of rat uterine preparation were present.

Dialysis of the uterine preparation did not remove the inhibitory effect.

Rat vagina:

In four experiments carried out with rat vagina, transhydrogenase activity was not found either in the absence or presence of oestradiol. Oestriol, which stimulates development of cervix, vagina and vulva more effectively than oestrone or oestradiol (see Merrill, 1958), is also without effect.

Hen oviduct:

The average weight of the oviduct from 5½ week old chicks in the present experiments was 33 mg., while the weight of this organ from oestradiol benzoate injected chicks rose to 1.8 g. Transhydrogenase activity could not be detected either in the control or oestrogen treated oviducts, nor did this enzyme activity appear after addition of oestradiol in vitro. When an oviduct preparation was mixed with the human placental enzyme, inhibition of the placental transhydrogenase occurs (Fig. II. 9).

Ox pituitary glands:

Experiments were performed with two separate samples of ox anterior pituitary glands and one with the posterior lobe. In neither case was

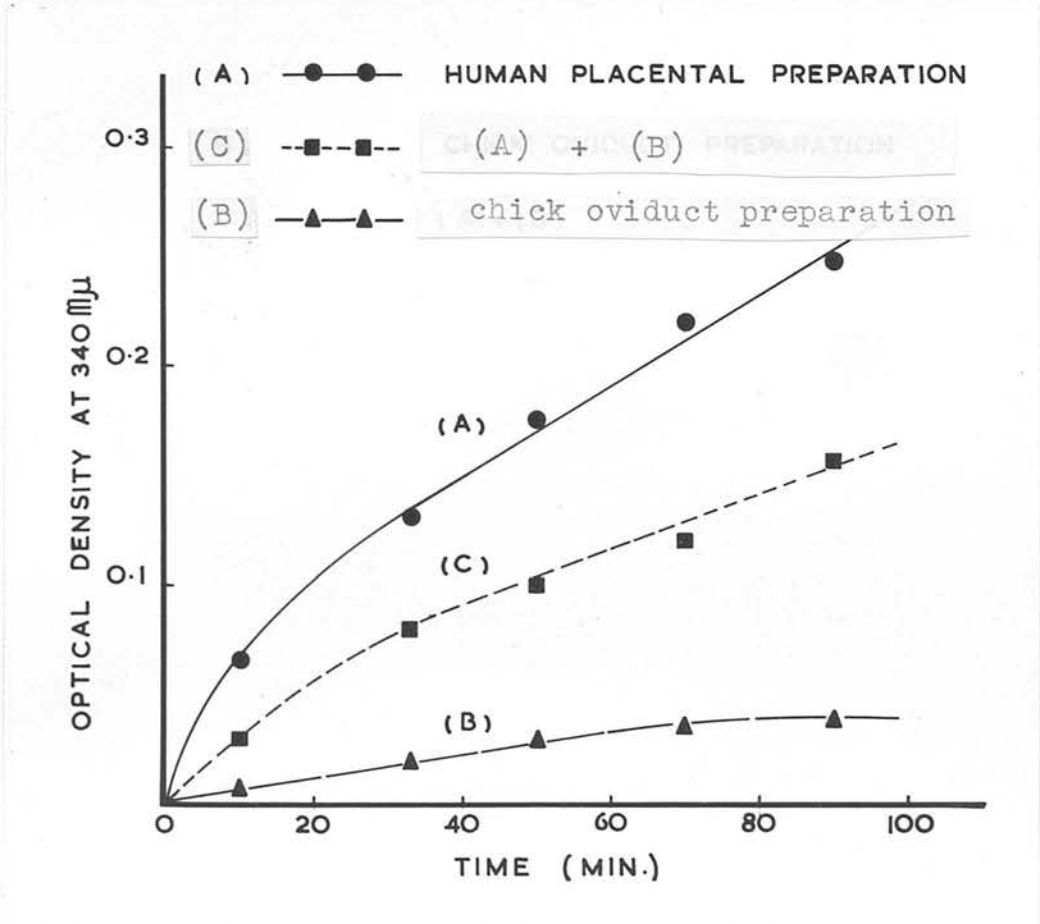


Fig. II. 9 . The inhibition of human placental oestradiol-activated transhydrogenase by the chick oviduct preparation.

transhydrogenase activity demonstrated and neither did oestradiol nor oestriol bring about the appearance of any activity.

(ii) Transhydrogenase activity in placentae of various species.

Preparations from the placentae of rat (1 experiment), rabbit (1 experiment), golden hamster (2 experiments), quinea-pig (4 experiments) and pig (1 experiment) were investigated. "Soluble fraction" transhydrogenase activity was not found in any of these tissues, either in the absence or presence of oestradiol or of oestradiol-17α in the case of the rabbit placental preparation. Typical results obtained with a preparation from hamster placenta are shown in Fig. II. 10. One positive result was obtained in the case of guinea-pig placenta, but this could not be repeated using either the spectrophotometric or the α-oxoglutarate method.

An inhibitory effect on the activity of the human placental transhydrogenase occurs when any of the inactive placental preparations are added (Fig. II. 11). The degree of inhibition depends on the amount of the latter preparation in the reaction mixture. The soluble fraction of a boiled guinea-pig

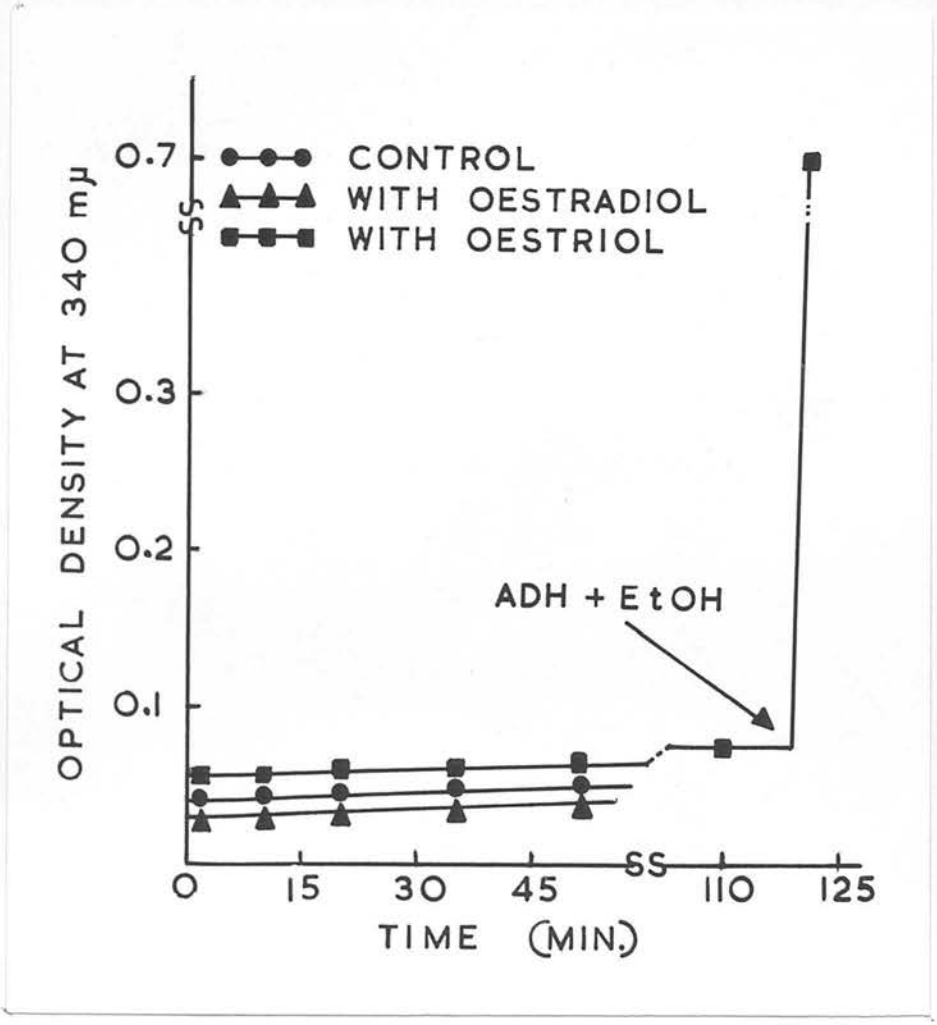


Fig. II. 10. The absence of transhydrogenase activity and lack of effect of oestrogens in hamster placental preparation. The increase in optical density after the addition of alcohol dehydrogenase and ethanol at 120 min. indicates that there was still an excess of DPN at the end of the incubation.

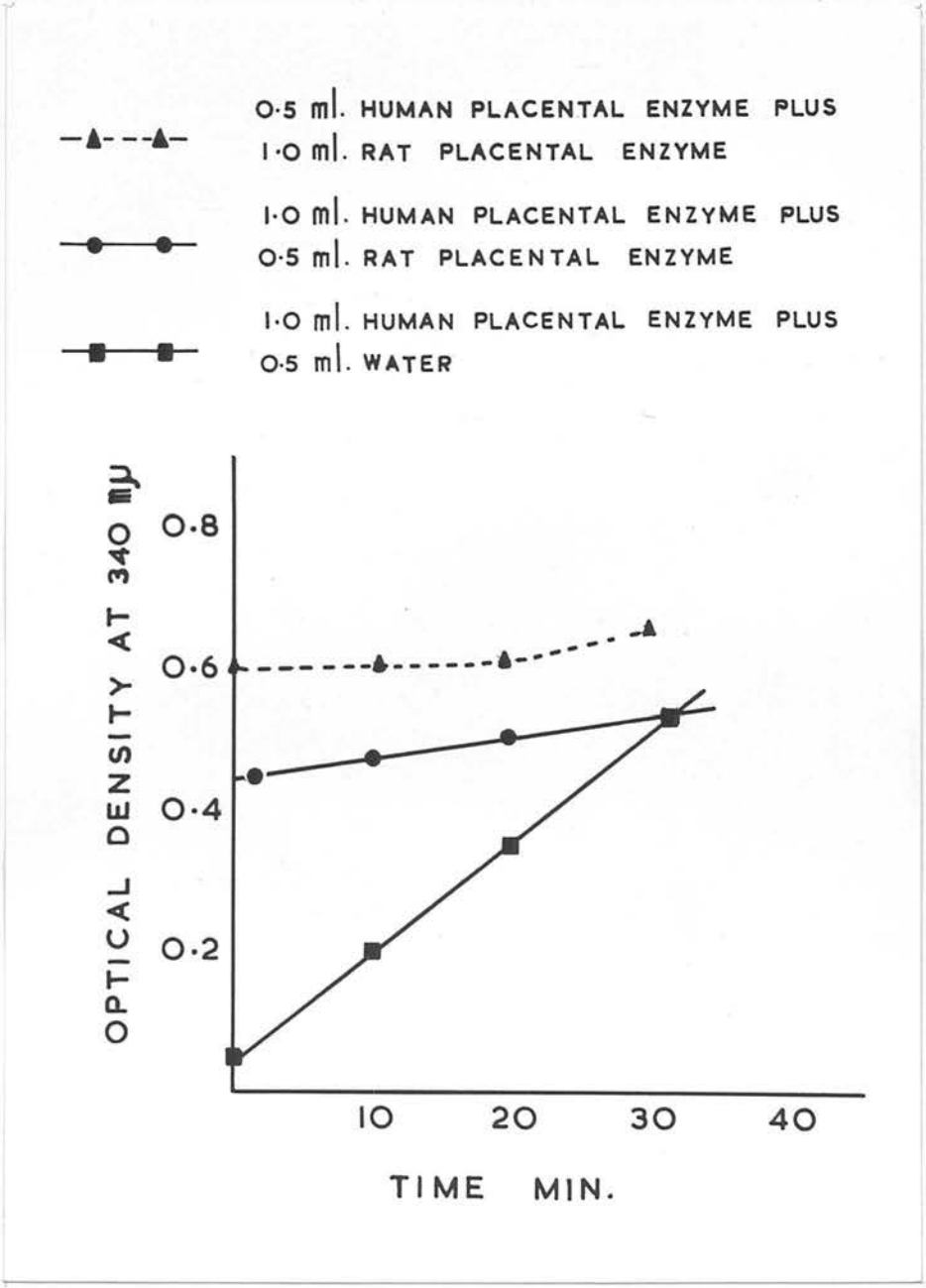


Fig. II. 11. The inhibiting effect of the rat placental preparation on the oestradiol-activated transhydrogenase of the human placental preparation.

placental preparation was found to be devoid of the inhibitory effect.

(iii) Other tissues.

Rat spleen and heart:

As observed by Humphrey (1957) only weak transhydrogenase activity was found in the soluble fraction of cells from rat heart and spleen. This was uninfluenced by added oestradiol or oestriol.

(c) Attempts to demonstrate steroid-activated transhydrogenase in microsomal and soluble fractions of cells from tissues containing hydroxysteroid dehydrogenases with dual pyridine nucleotide specificity.

Rat liver:

Only negligible transhydrogenase activity was found in microsomal and soluble fractions of rat liver cells. Addition of the 11β-hydroxysteroid, cortisol, was without effect.

Human, ox and rat adrenal cortex:

It has not been possible in the present investigation to demonstrate transhydrogenase activity in either the microsomal or the soluble fraction of adrenocortical tissue from rat, ox or human subjects. Cortisol (11β-hydroxysteroid), pregnenolone (3β-hydroxysteroid) and oestradiol (17β-hydroxysteroid) were

without effect. A typical result with added oestradiol is shown in Fig. II. 12.

(d) Mitochondrial transhydrogenase in the adrenal glands.

On repeating Scott & Lisi's experiments (Scott & Lisi, 1960) an active transhydrogenase was found in the supernatant fluid, obtained on centrifuging rat adrenal homogenate at 2,200 g. for 1 hr. However, when the preparation was centrifuged further at 20,000 g. for 1 hr., the transhydrogenase had completely disappeared (Fig. II. 13). Furthermore, when another preparation of rat adrenal homogenate was subjected to differential centrifugation, the transhydrogenase activity was found to reside in the mitochondrial fraction only, and not at all in the microsomal plus the soluble fractions. The mitochondrial enzyme is stimulated neither by oestradiol nor by cortisol. No transhydrogenase activity was found in the supernatant fluid of a rat adrenal medulla homogenate centrifuged at 2,200 g. for 1 hour.

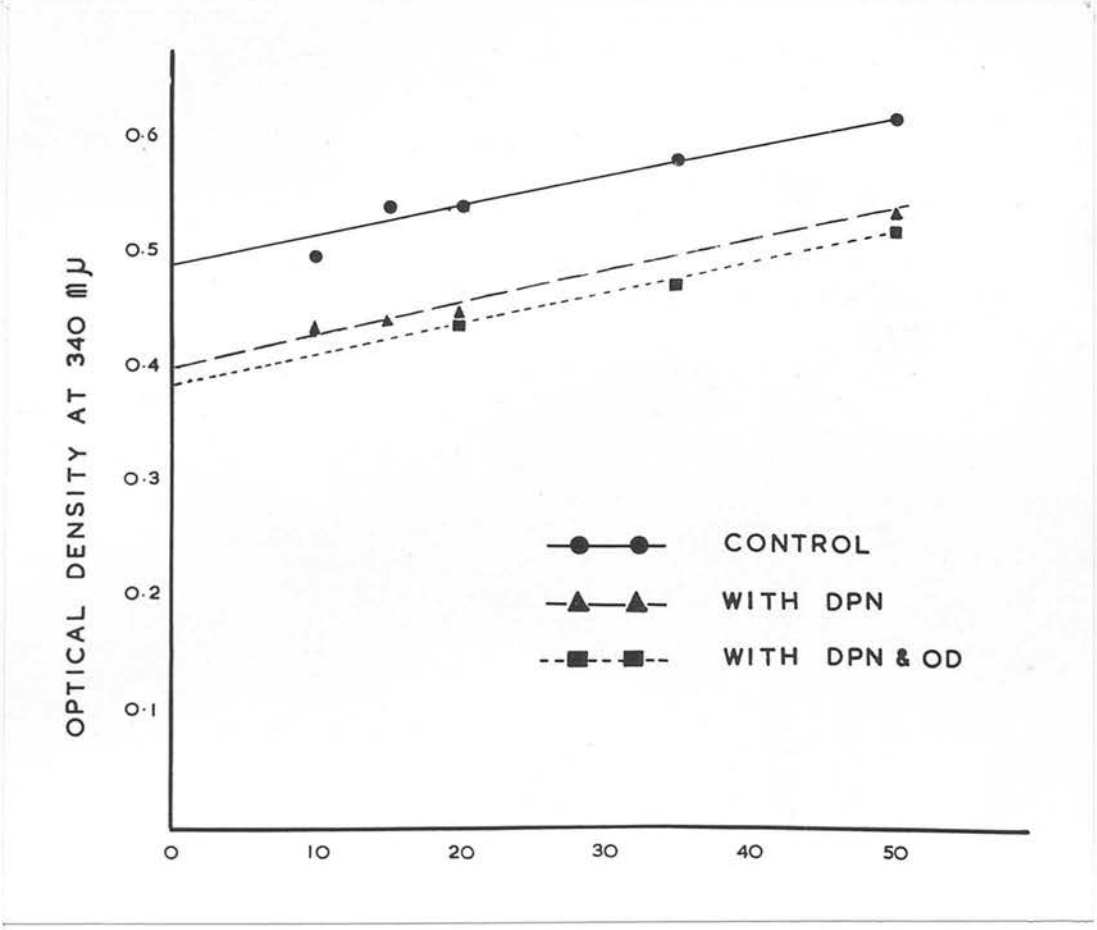


Fig. II. 12. The absence of transhydrogenase activity in the soluble fraction of an ox adrenocortical preparation. The slight nonspecific increase in the optical density occurred even when DPN was not present. Oestradiol is without effect on the system.

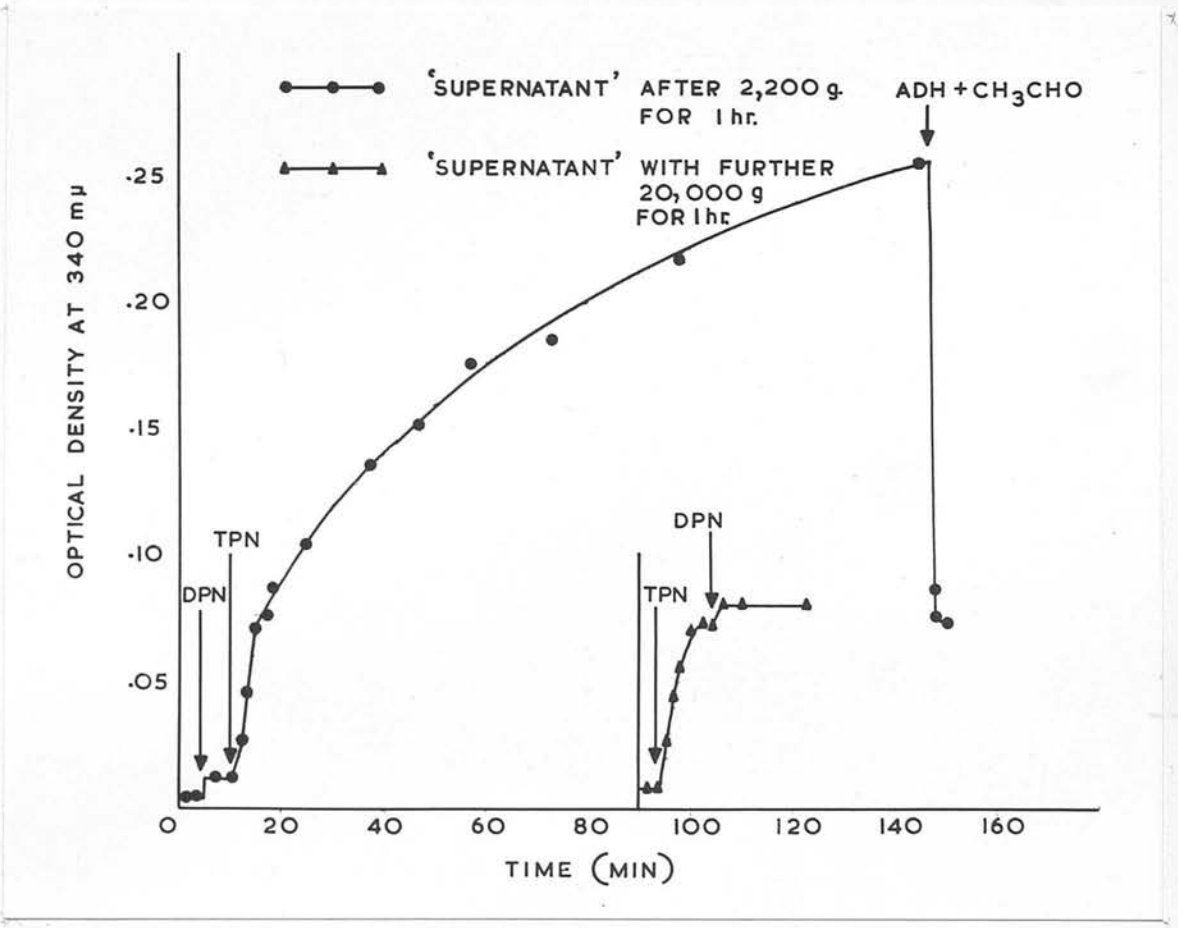


Fig. II. 13. Demonstration of transhydrogenase activity in the rat adrenal preparation obtained after centrifuging the 20% homogenate at 2,200 g. for 1 hr. However when this preparation was centrifuged further (20,000 g. 1 hr), the transhydrogenase activity disappeared completely. The 3 ml. final volume incubation mixture contained 50 mM tris buffer, pH 7.4; 1.7 mM G6P; 0.1-0.2 ml. adrenal preparations. 0.25 mM DPN and .01 mM TPN were added when indicated. The amount of TPN used, when fully reduced, corresponds to an optical density of 0.06 unit at 340 mμ. The blank cell contained no pyridine nucleotides. Alcohol dehydrogenase and acetaldehyde were added at the end of the experiment to demonstrate the presence of DPNH.

DISCUSSION

All attempts to demonstrate the presence of a soluble oestrogen-activated transhydrogenase in the well known oestrogen 'target' tissues of many animals have been negative. DPN and substrate (glucose-6-phosphate, or isocitrate) were always shown to be present in excess at the end of each negative transhydrogenase determination, as seen by the sharp rise in the optical density at 340 mμ when alcohol and alcoholdehydrogenase, or TPN were added to the incubation mixture. This addition of TPN did not, however, stimulate any DPNH production. It is, therefore, unlikely that the absence of transhydrogenase activity observed in the present investigation was due to the destruction of substrate or coenzymes during the experiment. The failure to stimulate transhydrogenase activity by oestrogen in the rat uterus, human endometrium, and the pituitary gland of the ox, even though increases in the oxygen consumption on adding oestrogen have been observed in these tissues, indicates that some other enzyme (or enzymes) is primarily activated by the oestrogen added in vitro. (The possibility that the oestrogen effect on oxygen consumption is a permeability phenomenon cannot, however, be excluded.) The enzyme or enzymes concerned may be those we already know, such as the enzymes of

carbohydrate, fat, or protein metabolism in the final part of which oxygen is being consumed via the citric acid cycle and the cytochrome system. On the other hand, it may be a new enzyme not yet familiar to us. The identification of the enzyme(s) concerned should merit further investigation as it would be of much value in the study of the mechanism of action of oestrogen. Transhydrogenase appears to be the key enzyme in the human placenta. The possibility of stimulating oxygen consumption in the rat vagina and chicken oviduct by oestrogen has not yet been investigated. Whether these tissues also possess the same type of oestrogen-sensitive enzyme as discussed above, is again a matter for further investigation.

Many of the effects of oestrogen on animal tissues, such as those shown by Mueller and co-workers (Mueller et al, 1958) and in the present investigation (SECTION IV), have been observed some hours after oestrogen administration in vivo, while there is no effect if the oestrogen is added in vitro. However, the absence of transhydrogenase activity in the chick oviduct from control and oestrogen treated birds indicates that this enzyme activity does not appear on treatment with oestrogen. (A further attempt to demonstrate the effect of oestradiol

administered in vivo on uterine transhydrogenation was made by studying the ratios of oxidised and reduced DPN and TPN. The results of this study are presented in SECTION III of this thesis).

The absence of an oestrogen-activated transhydrogenase in the placentae of rat, guinea-pig, hamster, rabbit and pig, confronts us with another example of species differences. These five species with no active transhydrogenase on the one hand and the human with an active enzyme on the other. The lack of stimulation by oestradiol-17α in the rabbit placenta is perhaps not unexpected in view of the inability of oestradiol-17β to stimulate transhydrogenation.

The inhibition of human placental transhydrogenase by the preparations from rat uterus, chick oviduct, and from placentae of rat, guinea-pig, rabbit and pig shows that the apparent absence of transhydrogenase in these tissues may be due to the presence of an inhibitor. Talalay, Hurlock & Williams-Ashman (1958) stress the importance of the DPN/TPN ratio in the incubation mixture used to study transhydrogenase activity, because high TPN concentration (above 10^{-5}M) inhibits the enzyme markedly. Hence TPN in a bound form (dialysis did not remove the inhibition) may be the inhibitor. However, the amount of

TPN in the oxidised and reduced state is found to be low in the rat uterus (see SECTION III of this thesis), in the rat placenta (Glock & McLean, 1955b) and in the human placenta (Villee, Joel, Loring & Spencer, 1960). In the 3 ml. incubation mixture containing either preparations of human placenta and rat uterus, or human and rat placentae, the total oxidised and reduced endogeneous TPN can be calculated to be between 1 and 5 X 10^{-6}M which is below the critical concentration of 10^{-5}M. Hence it is unlikely that the inhibition of the human placental transhydrogenase is caused by the endogeneous triphosphopyridine nucleotide in the preparations. Since the inhibitory property of guinea-pig placentae preparation was lost after boiling, the nature of inhibition may be enzymic. The fact that dialysis does not remove the inhibitory effect in these preparations also indicates that large (protein ?) molecules may be involved in the inhibiting process. The nature of the inhibitor(s), is at present unknown. This is another interesting topic for future investigation.

The observation that 2-fluoro-oestradiol-17β is as effective as oestradiol in the stimulation of human placental transhydrogenase rules out the possibility that a 2-hydroxy derivative is the active substance in this system.

Substances antagonising oestrogenic responses *in vivo* might well be expected to inhibit oestrogen action *in vitro* especially if these *in vitro* systems are of physiological significance. However, well known oestrogen antagonists such as MER-25 and amphenone-B are found to be without inhibitory effect upon the activity of oestradiol-activated placental transhydrogenase. The lack of oestrogen antagonising effect of amphenone-B may, however, be explained by the fact that this compound was found by Hertz (1958) to interfere with corticosteroid biosyntheses in the adrenals resulting in the production of progesterone-like substances, which may be the true *in vivo* oestrogen antagonists.

In general it would appear that there is negligible transhydrogenase activity in the soluble fraction of cells of the tissues studied. The active transhydrogenase found in the 'supernatant' of rat adrenal preparation by Scott & Lisi (1960) has been shown here (p. 39) to be of mitochondrial origin.

The inability of an 11-hydroxysteroid (cortisol) to stimulate transhydrogenation in the rat liver, rat and ox adrenal glands, and of a 3β-hydroxysteroid (pregnenolone) to have the same effect in the rat and ox adrenal glands, although these tissues possess the relevant steroid dehydrogenase activity, shows that these steroids do not

in fact function as coenzymes as suggested by Talalay and co-workers. Such observations lend support to the view that the oestrogen-activated transhydrogenase and the oestradiol dehydrogenase in human placenta are indeed separate and distinct enzymes. This idea has been advanced by Villee and co-workers (Villee, Hagerman & Joel, 1960) who recently summarise a long list of the differences in the properties of the two enzymes. Besides numerous differences towards inhibitors and heat inactivation, the most convincing evidence is that the transhydrogenase and dehydrogenase activities can be separated by electrophoresis on a starch block and by continuous flow paper curtain electrophoresis (Hagerman & Villee, 1959). Therefore, judging from present evidence, Talalay's coenzyme theory where steroids are visualised as hydrogen carriers similar to the concept of the functions of some vitamins, cannot be accepted without further modification or new supporting evidence. This is rather unfortunate because the speculation is the first of its kind to offer an explanation of how a hormone may work at molecular level.

A fuller discussion of the transhydrogenase theory and the mechanism of action of oestrogens in the light of results reported in this Section and in Section III is to be found in the General Discussion (SECTION VI).

SECTION III

THE EFFECT OF OESTRADIOL ON THE CONCENTRATIONS OF OXIDIZED AND REDUCED DPN AND TPN IN THE RAT UTERUS.

INTRODUCTION

In SECTION II an attempt was made to demonstrate transhydrogenase activity in oestrogen-sensitive tissues and to show an *in vitro* effect of oestrogen on this enzyme. Failure to demonstrate this enzyme activity might be due to its loss during handling of the tissues, to an unsatisfactory assay procedure for the particular tissue concerned or to the presence of an inhibitor. An alternative approach might be to attempt to show changes in the proportions of oxidised and reduced DPN and TPN in the tissues which might be due to the effect of transhydrogenase influenced by oestrogen administered *in vivo*. It is possible to compare the ratios DPN/DPNH and TPN/TPNH in oestrogen sensitive tissues from castrated animals with and without administration of oestrogen. A transfer of hydrogen from DPNH to TPN would cause an increase in DPN/DPNH with a corresponding decrease in TPN/TPNH and similarly a transfer of hydrogen from TPNH to DPN would cause the opposite change in the ratios. Either change might be taken as an indication of transhydrogenase activity. Thus a possible effect of oestrogen might be investigated by a study of changes in these ratios.

The influence of hormones on pyridine nucleotide concentrations is considered in the General Introduction (p. 9-10). It is of interest to find if oestrogens have

any effect on the concentrations of these important coenzymes in the 'target' organs. This information may be useful in understanding how the hormone works in these tissues.

PLAN OF INVESTIGATIONS

The rat uterus, by virtue of its dramatic response to oestrogen administered in vivo (see Fig. I. 1), was chosen for this investigation.

It was necessary to determine the concentrations of DPN, DPNH, TPN and TPNH in the uteri of rats belonging to the following groups:-

(i) 'normal'* or intact

(ii) ovariectomised

(iii) ovariectomised and injected with oestradiol

In this way it was hoped to compare the changes, if any, in the concentrations of the four forms of coenzymes following castration, and after treatment of castrated animals with oestrogen.

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* Intact rats used in the present investigation (Sections II, III, IV and V) were chosen at random, without any attempt to establish the phases of their oestrous cycle. It was assumed that, with a reasonable number of animals, values (for pyridine nucleotide concentrations, enzyme levels, etc.) obtained for this group of animals can be taken as the mean values for animals between the two extreme phases, oestrus and dioestrus. These mean values (described as

'normal' rat values), together with their variations, are assumed to represent the ranges of levels of enzymes and coenzymes, etc. normally occurring in the average intact rats. At most, however, they can be considered only as a guide when comparing with values obtained from animals artificially created as oestrus and dioestrus by ovariectomy with and without oestrogen administration.

EXPERIMENTAL

Animals and Operations

The white Wistar rats used were 3 to 6 months old and weighed about 200 g. They were ovariectomised under ether anaesthesia and left 2-4 weeks before experiments. Small pieces of uterine horns were removed with the ovaries to ensure complete ovariectomy.

Injections

For subcutaneous injections, oestradiol was dissolved in small amount of ethanol (1 mg/ml) and diluted with sesame oil to give the concentrations required. Animals were given three daily injections of 1 µg oestradiol in 0.1 ml volume, and they were killed 72 hours after the first injection. For intravenous administration a solution of 100 µg oestradiol/ml was prepared in physiological saline according to Roberts & Szago (1947). In each case a single dose of 10 µg was injected in a volume of 0.1 ml into either the tail, femoral or jugular vein. The animals were killed after periods stated in text.

Preparation of tissues

Rats were killed by breaking their necks and the uteri quickly removed following an incision at the fundus.

Thus uterine horns but not corpus were used in the experiments. Adhering fat and connective tissues were removed with the uterus lying on a tile chilled on ice. The uterus was slit open lengthwise with scissors, blotted with filter paper and weighed in a precision type torsion balance. A piece of the uterine tissue chosen at random was reserved for dry weight determination.

Dry weight determination

Pieces (about 20-25 mg) of uterine tissue were blotted, weighed on a torsion balance (correct to 0.2 mg) and placed on a small piece of dried, weighed tin foil kept in a desiccator. It is important to weigh the uterus very soon after removing fat and connective tissues since water from the small piece of tissue evaporates considerably even when the tissue is sitting on ice. The tissue on tin foil was then left in an oven at 110° for 24 hours, at the end of which it was allowed to cool to room temperature in a desiccator before being reweighed. The percent dry weight of the uterus was then calculated.

Selection of the method of determination of the pyridine nucleotides

DPN, discovered by Harden & Young (1906) and

TPN by Warburg & Christian (1931), have been shown to occupy a special place among the catalysts of biological oxidation. While these substances have been isolated and purified, and their structure and biochemical roles determined, a convenient and satisfactory method of measuring the oxidised and reduced forms in animal tissues has yet to be found. An apparently reliable, if rather laborious, method has been available since 1955 (Glock & McLean, 1955a). In this enzymic method the reduced coenzymes are coupled with their respective cytochrome c reductases and the rate of reduction of a pool of cytochrome c is followed spectrophotometrically. As little as 3×10^{-7}M can be measured by this method.

A second method by Jacopson & Astrachan (1957) is based on the measurement of fluorescence of the pyridine nucleotides developed by concentrated alkali solution. The lowest concentration that can be measured is reported to be 10^{-8}M.

Bassham, Birt, Hems & Loening (1959) developed another fluorimetric method for the determination of the oxidised and reduced pyridine nucleotides with sensitivity down to 1.5×10^{-6}M or lower. The advantage of this fluorimetric method, over that of Jacopson & Astrachan (1957), is that the preparation or expensive purchase of some five enzymes is avoided.

After the present investigation was finished, a fourth method, a modification of that of Glock & McLean (1955a), was presented by Villee (1961). In this, the difficulty of the preparation of DPNH- and TPNH-cytochrome c reductases is overcome by the use of commercially available diaphorase and the reduction of 2:6 dichlorophenolindophenol instead of the more expensive cytochrome c is measured. However the full procedure and the sensitivity of the method have yet to be published.

At the beginning of this investigation much time was devoted to finding a suitable method for the extraction and determination of oxidised and reduced DPN and TPN. The method of Glock & McLean (1955a) was tried first. TPNH-cytochrome c reductase was prepared from Younger's Top Ale brewer's yeast by the method of Haas, Horecker & Hogness (1940) as modified by Glock & McLean (1955a). A darkish-brown precipitate was obtained and found to contain 2 units/mg of the reductase activity. It was devoid of DPNH-cytochrome c reductase activity. The enzyme was quite stable at -15°. The preparation of DPNH-cytochrome c reductase from pig hearts followed the method of Mahler, Sarkar, Vernon & Alberty (1952) modified by Glock & McLean (1955a). Although Hems & Villee (private communications) both had much difficulty in the preparation

of this enzyme, a fairly active preparation containing 12-16 units/ml was obtained. This, however, was rather unstable. In one case, only 10% of the enzymic activity remained after 5 weeks storage at -15°. Owing to the very tedious preparation of the enzyme and its instability this procedure is rather unattractive.

Another difficulty that arose unexpectedly, was that some impurity in the cytochrome c used (purchased from Lights & Co.) was precipitated by rat tissue extracts, especially with the alkali extract. This interfered seriously with accurate optical density measurements. The problem was solved by using a more expensive but purer preparation of cytochrome c obtainable from Sigma & Co., or could possibly have been solved by direct preparation of the substance from horse heart (Keilin & Hartree, 1952, as modified by Margoliash, 1954). These solutions are again not too attractive due to the high cost of the purchased material or of time and effort needed to prepare the highly purified material, or to recover it, as suggested by Glock & McLean (private communication). In view of these difficulties this method was not proceeded with.

Method finally chosen

The method of Bassham, Birt, Hems & Loening

(1959) was then tried in preference to that of Jacopson & Astrachan (1957) in view of the fact that many enzymes needed to be prepared in the latter method. Since they are based on the same principle of measuring fluorescence produced on incubating the coenzyme with concentrated alkali, the sensitivity of the two procedures should be comparable. The method of Bassham et al (1959) was found to work satisfactorily in the author's hands and therefore it was employed in the determination of pyridine nucleotides in the rat uterus with a few minor modifications.

Only an outline of the procedure, with the modifications adopted, is given here.

Extraction of coenzymes:

It was found easier to grind tough uterine tissue in conical shaped glass homogenisers with conical pestles in place of the usual cylindrical type of apparatus (Potter & Elvehjem, 1936). Two homogenisers with pestles were heated in a boiling water bath for at least 5 minutes. One contained the "acid medium" (2.5 ml of 0.1 M 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer pH 8.2, 1 ml H_2O and 0.5 ml N.HCl) for extracting DPN and TPN, the other the "alkaline medium" (2.5 tris buffer, 1 ml H_2O and 0.5 ml N.NaOH for extracting DPNH and TPNH. Portions of uterus,

sometimes from a pool of tissue, weighing 100-200 mg were dropped into the hot extraction solutions. After 30 seconds the homogenisers were removed from the bath, the contents ground for 1½ minutes, while still hot, and then immersed in a bath of acetone-solid CO_2 until the contents were frozen. The contents were subsequently thawed, pH adjusted to 8.2 and volumes to about 5.0 ml. After centrifuging for 15 minutes at 0° and 20,000 g. the clear supernatant solutions (referred to as the "Acid" and "Alkaline Extracts") containing the pyridine nucleotides were removed and treated further without delay.

Fluorimetric measurement:

Solutions of glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), alcohol dehydrogenase (ADH) and approx. N-HCl and N-NaOH were prepared according to Bassham et al (1959). On account of the low pyridine nucleotide content of the rat uterus, it was found necessary to modify the sodium hydroxide-hydrogen peroxide solution in order to minimise the dilution of the pyridine nucleotide solution. This solution was prepared immediately before use by adding 0.16 ml 3% (W/V) hydrogen peroxide to 3.0 ml 15 N-NaOH. A standard solution of 3 µM DPN was prepared in 0.03 M tris buffer pH 8.2.

A 2 ml volume of the "Acid Extract", containing DPN and TPN was mixed with 0.05 ml G6PDH and 0.05 ml G6P and kept at room temperature for 20 minutes. The product (Solution A) now contains DPN and TPNH.

A 2 ml volume of the "Alkaline Extract", containing DPNH and TPNH, plus small amounts of DPN and TPN, was mixed with 0.15 ml N. HCl, kept for 5 minutes at 60°, cooled and pH adjusted to 8.2. The resultant solution containing small amounts of DPN and TPN was then treated with G6PDH and G6P as described in the previous paragraph. The product (Solution B) now contains DPN and TPNH.

A second 2 ml volume of "Alkaline Extract" was mixed with 0.15 ml N-NaOH, kept for 15 minutes at 60°, cooled and adjusted to pH 8.2. The resultant solution, containing DPNH & TPNH, was mixed with 0.05 ml 0.03 M ethylenediaminetetra-acetate (EDTA), 0.01 ml alcohol dehydrogenase and 0.04 ml 0.0075 M acetaldehyde and kept at room temperature for 20 minutes. The product (Solution C) now contains DPN and TPNH.

In subsequent stages of the analysis Solutions A, B and C were treated similarly. Eight 0.25 ml volumes of each solution were measured into tubes for use in the Farrand Fluorimeter - Model A (Farrand Optical Co. Inc., New York). To one pair of tubes (a) was added 0.05 ml

water, to a second (b) 0.02 ml N. HCl + 0.03 ml water, to a third (c) 0.02 ml N. HCl followed after 5 minutes by 0.07 ml N-NaOH and to a fourth (d) 0.05 ml N.NaOH. The contents of all tubes were mixed and tubes (c) and (d) containing added alkali were heated at 60° for 15 minutes and cooled. A 0.21 ml volume of the NaOH-H_2O_2 solution was added to all tubes. After mixing the tubes were incubated for 1 hr. at 38° and the contents finally diluted with 2 ml water. The fluorescence developed was measured in the fluorimeter with primary filter Farrand No. 5860 and secondary filters Farrand No.3389 and 4308 and Wratten No. 2B. The instrument was standardised against a solution of 0.005 µg/ml quinine sulphate in 0.1 N H_2SO_4 each time before use.

Range of the proportionality of the amount of coenzyme and degree of fluorescence

As shown in Fig. III. 1 the relationship between the fluorimeter galvanometer readings and the concentrations of pyridine nucleotides in the solution is linear up to a concentration of 1 mµmole per tube, above which the curve starts to flatten out. The amount of the uterine extract taken for a determination was therefore selected to ensure that this concentration was not exceeded.

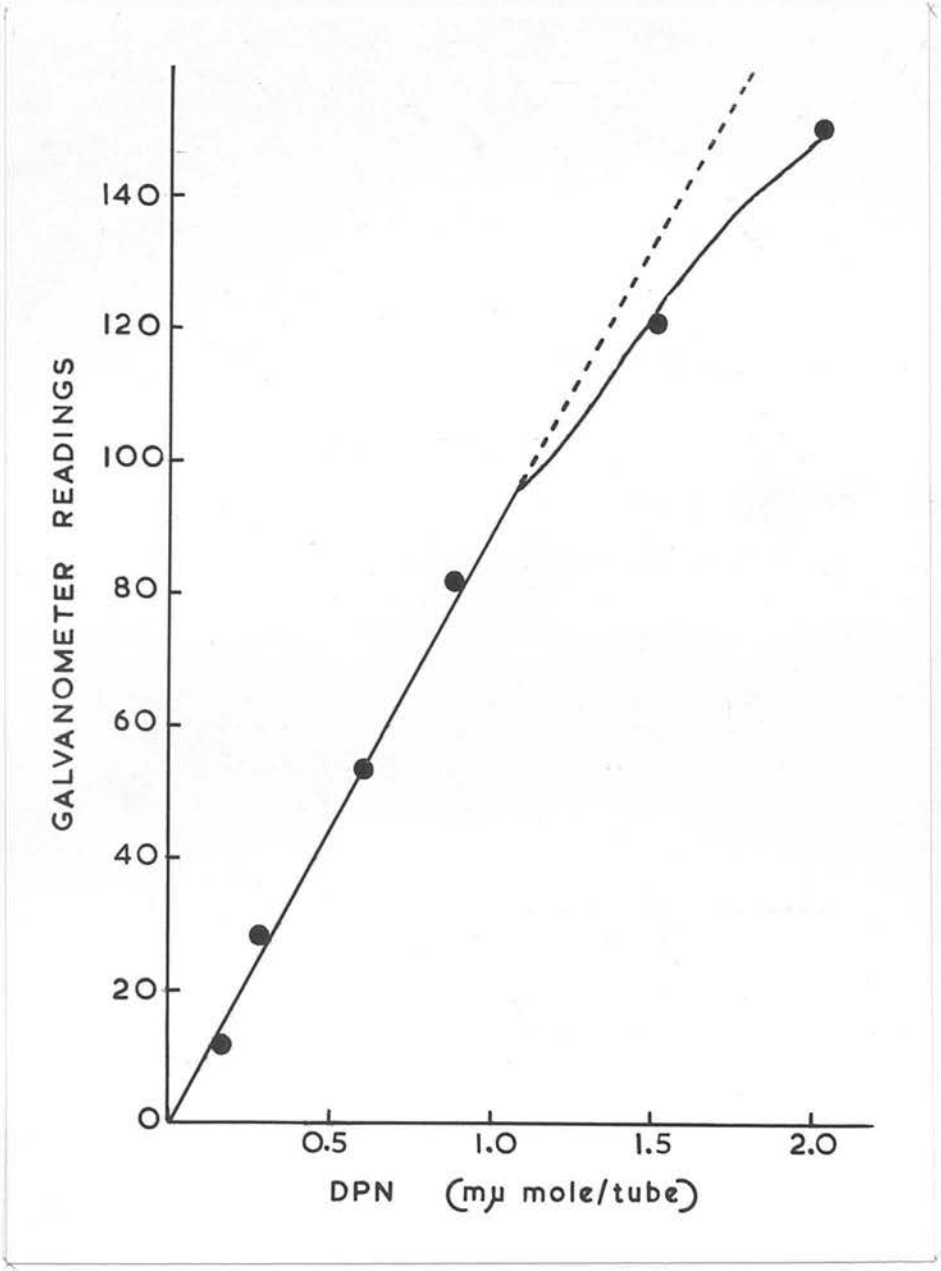


Fig. III. 1. Relationship between amounts of DPN and degree of fluorescence developed.

Recovery of added coenzymes

The recovery of DPN, DPNH, TPN and TPNH was determined after adding known amounts of these substances to the hot extracting media alone and to the hot media during extractions of uterine tissue. The native coenzymes were determined separately. The results are shown in Table III. 1. In the author's hands, the method gives slightly lower recovery of the nucleotides than was reported by Bassham et al (1959), who worked with liver. However, here much lower concentrations of pyridine nucleotides of the rat uterus (as compared with the high coenzyme concentrations of the rat liver) were under consideration. As seen in Table III. 1 high recovery of coenzyme was obtained when only extracting media were used as compared with that when uterine tissue was present. This may be due to the presence of substances which tend to quench the fluorescence of nucleotides slightly. Hems (personal communication) also experienced this quenching effect in the liver extracts. It is only logical that, the lower the concentration of nucleotides in the solution (as in the case of the uterus) the more marked is the quenching effect.

Table III. 1.

Recovery of pyridine nucleotides added to either extracting medium alone, or to uterine preparation during extraction. Values are means with Standard Error of Means (S.E.M.). Numbers in parentheses indicate numbers of experiments.

Coenzymes	mμ mole of coenzyme added to the extracts	Recovery from extracting medium (%)	Recovery from uterine preparation (%)
DPN	20 - 30	104 (1)	90 ± 6.8 (9)
TPN	3 - 14	90 (1)	76 ± 6.0 (9)
DPNH	10 - 30	84 ± 2.0 (7)	80 ± 12.7 (5)
TPNH	10 - 25	88 ± 2.7 (7)	76 ± 13.1 (5)

RESULTS

The concentrations of oxidised and reduced pyridine nucleotides were determined in the uteri from ovariectomised rats untreated and after administration of oestradiol either subcutaneously or intravenously. Uteri from intact 'normal' animals, (killed at random stages of the oestrous cycle) were also analysed for comparison. Results summarised in Table III. 2 and Fig. III. 2 show concentrations of pyridine nucleotides in the rat uteri expressed per unit wet and dry weight respectively. There is no apparent marked difference between results expressed in either of these ways, although there is some difference in the water content of the uteri in the different groups of animals (see Table III. 3). In general, DPN and TPN concentrations in uteri of castrate rats are not significantly different from those in oestrogen-treated animals nor from those of 'normal' rats. The concentrations of oxidised coenzymes seem to fluctuate, without any recognisable pattern, within the limits of experimental error. On the contrary, changes in the reduced pyridine coenzymes follow a definite pattern. Thus both uterine DPNH and TPNH concentrations in the castrate rats decrease 6 hours after oestrogen administration, but start to rise again by the 12th hour. The increase continues up to 36 hours after the

Table III. 2.

Concentrations of pyridine nucleotides in the rat uterus in mμ moles per g. wet weight (with S.E.M.)

Types of rats	DPN	DPNH	DPN/DPNH ratio	TPN	TPNH	TPN/TPNH ratio	No. of experiments
'Normal'	69 ± 5	21 ± 3	3.3	9 ± 1	24 ± 4	0.38	10
OVX*	74 ± 4	17 ± 1	4.4	8 ± 0.2	19 ± 2	0.42	18
OVX, 6 hrs. after OD** (i.v.) injection	67 ± 9	10[a] ± 1	6.7	6 ± 1	12 ± 1	0.50	5
OVX, 12 hrs. after OD (i.v.) injection	65 ± 7	14 ± 2	4.7	9 ± 1	14 ± 2	0.64	5
OVX, 24 hrs. after OD (i.v.) injection	54 ± 7	16 ± 2	3.4	8 ± 1	18 ± 3	0.45	7
OVX, 36 hrs. after OD (i.v.) injection	81 ± 8	17 ± 2	4.8	8 ± 1	18 ± 1	0.45	4
OVX, 72 hrs. after three daily OD (s.c.) injections.	69 ± 5	27[b] ± 3	2.6	6 ± 0.3	22[c] ± 2	0.27	8

* OVX = ovariectomised

** OD = oestradiol

[a]: $0.02 > p > 0.01$ (c.f. OVX value)

[b]: $0.001 > p$ (c.f. OVX value)

[c]: $0.01 > p > 0.001$ (c.f. OVX, 6 hrs. after OD (i.v.) injection).

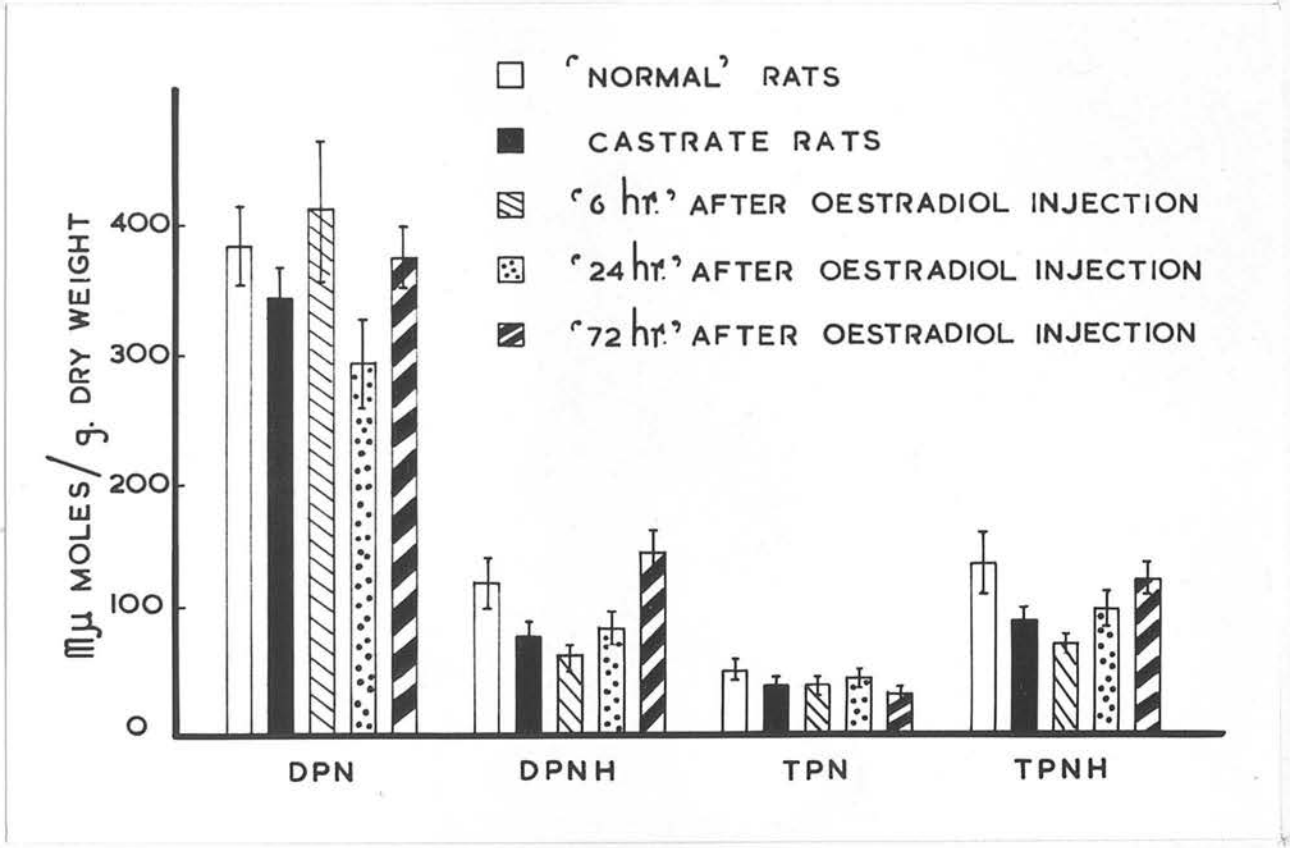


Fig. III. 2. Concentrations of various forms of pyridine nucleotides in the rat uterus. '6 hr' and '24 hr' indicate hours after 10 μg oestradiol intravenous injection to the animals. '72 hr' indicates the hour after three subcutaneous 1 μg oestradiol injections done at 0, 24 and 48 hours.

Table III. 3.

Percentages dry weight of uteri (with S.E.M.) from rats of different oestrogenic status.

Types of rats	% dry weight	No. of experiments
'Normal'	18.0 ± 0.65	4
OVX*	21.5 ± 0.36	10
OVX, 6 hrs. after OD** (i.v.) injection	16.1 ± 0.62	4
OVX, 24 hrs. after OD (i.v.) injection	18.6 ± 0.38	3
OVX, 72 hrs. after OD (s.c.) injection	18.6 ± 0.52	3

*OVX = ovariectomised

**OD = oestradiol

administration of oestrogen. Furthermore, when oestradiol was administered daily to ovariectomised rats for 3 days, DPNH concentration at the 72nd hour from the first oestrogen injection is very markedly higher than the concentration in the untreated ovariectomised animals. There is only a slight increase in TPNH concentration at a similar time. Both DPNH and TPNH levels in the 'normal' rats are also higher than the ovariectomised rat values. (Only the decrease of DPNH concentration after 6 hours, and the increase of DPNH concentrations after 72 hours of oestrogen treatment are significantly different from the values in the untreated ovariectomised rats).

Since the oxidised coenzyme levels are almost constant, the increase and decrease in the DPN/DPNH and TPN/TPNH ratios depend largely on the concentration of reduced coenzymes. Thus the DPN/DPNH and TPN/TPNH ratios appear to increase slightly up to 36 hours from the time of oestradiol administration as compared with the untreated ovariectomised animals. Both coenzyme ratios are markedly lowered in the animals receiving oestrogen daily over three days and killed on the fourth. The two ratios are also lower in the 'normal' rats than in the spayed rats (Table III. 2).

The uterine weight dropped markedly after

ovariectomy (Table III. 4), and increased gradually as a result of growth following oestradiol administration. When the quantities of the di- and triphosphopyridine nucleotides in the rat uterus are expressed as mμ moles per gland, as in Table III. 4 it is seen that they decrease sharply as an effect of ovariectomy and increase gradually towards the 'normal' rat values following oestrogen administration. Both uterine weight and pyridine nucleotide concentration show increases of the same order following administration of oestrogen to the ovariectomised animals.

Table III. 4.

Rat uterine wet weight (with S.E.M.) and the amount of pyridine nucleotides per gland. The relative changes (taking values for castrate rats = 100) are also shown.

Types of rats	Uterine weight		DPN + DPNH		TPN + TPNH	
	actual wt. (mg.)	OVX value = 100	mμ mole/gland	OVX value = 100	mμ mole/gland	OVX value = 100
'Normal'	494 ± 51	500	44.4	500	16.3	600
OVX*	99 ± 3	100	9.0	100	2.7	100
OVX, 6 hrs. after OD** (i.v.) injection	113 ± 15	114	8.7	97	2.0	76
OVX, 12 hrs. after OD (i.v.) injection	166 ± 18	168	13.1	146	3.8	141
OVX, 24 hrs. after OD (i.v.) injection	131 ± 18	132	9.2	102	3.4	128
OVX, 36 hrs. after OD (i.v.)injection	144 ± 11	146	14.1	157	3.7	141
OVX, 72 hrs. after three daily OD (s.c.) injections	240 ± 14	242	23.0	257	6.7	248

* OVX = ovariectomised

** OD = oestradiol

DISCUSSION

The relative amounts of DPN, DPNH, TPN and TPNH in the rat uterus are found to be similar to those in other tissues (see Glock & McLean, 1955b). Thus diphosphopyridine nucleotide, mostly in the oxidised form, is in much greater concentration than triphosphopyridine nucleotide, the majority of which is in the reduced form.

The concentrations of the coenzymes in the rat uterus are however in general much lower than in the liver or adrenal gland but are within the ranges of many other tissues (see Glock & McLean, 1955b). The low concentrations of the coenzymes, however, do not reflect inactivity of the tissue since the concentrations in the proliferating uterus are not much greater than those in the atrophic gland. It would appear that relatively low concentrations of coenzyme suffice for the 'normal' range of metabolic activities in this tissue. Perhaps this is not unusual. In some *in vitro* systems such as the oestrogen-activated transhydrogenase system only a minute catalytic amount of TPN (5×10^{-8}M) is necessary and higher concentrations such as 10^{-5}M are inhibitive (Talalay *et al*, 1958).

Ovariectomy and subsequent oestrogen treatment have little effect on the concentrations of *oxidised*

coenzymes in the uterus, but the effect on the concentrations of reduced coenzymes are more pronounced, especially in the case of DPNH. The DPNH concentration, 72 hours after oestrogen administration, is significantly higher than the castrate rat levels and the trend of the change in TPNH concentration is similar. These data suggest that an ultimate action of oestrogen in vivo is to lower the DPN/DPNH and TPN/TPNH ratios.

The fact that both of these ratios change in the same direction diminishes the likelihood that transhydrogenation is involved as a result of oestrogen action in the tissue (see p.46). This would be in keeping with the report in SECTION II that no significant transhydrogenase activity could be detected in the rat uterus in the absence or presence of oestrogen. However, the increased concentrations of DPNH and TPNH, no matter how brought about, may reflect the demand for energy (from the former) and specific reductant (from the latter) for biosynthesis occurring in response to oestrogen.

The marked decreases in DPNH and TPNH levels, 6 hours after oestrogen treatment, and their increases thereafter reaching highest values by the 72nd hour, may indicate changes in the rate at which the two reduced coenzymes are produced and utilised. An increased rate of

re-oxidation of DPNH and TPNH coupled with a lesser or non-increased rate of production of the two reduced coenzymes would result in the lowering of their concentrations in the uteri (e.g. 6 hour levels). Similarly, on the other hand, a much greater acceleration of the rate of production of reduced coenzymes, without a corresponding increase in the rate of their utilisation, would result in an increase in DPNH and TPNH concentrations (e.g. 72 hour levels). There is evidence in support of this theory. Thus rapid water imbibition in the rat uterus is known to occur following oestrogen administration. This attains a maximum between 4 and 6 hours after giving oestrogen (Ashwood, 1938; see also Table III. 3). During this period, oxygen consumption and glucose utilisation also increase (Szego & Roberts, 1953). These observations suggest that between 4 and 6 hours after oestrogen injection, much energy is being consumed, such as in transportation of water into uterine tissue and possibly also in biosynthetic processes, especially those of ribonucleic acid and protein which are accelerated at this stage (Mueller et al, 1958). These processes would involve an increase in the oxidation of DPNH and TPNH (see p. 7). The uterine levels of some DPN- and TPN-linked dehydrogenases are found to be unchanged at this early stage of oestrogen treatment (Mongkolkul & Grant, 1960; see also

SECTION IV, Fig. IV. 1). Assuming these pyridine nucleotide reducing enzymes are rate limiting and cannot keep up with the increased reoxidation processes discussed above, it is possible to imagine a decrease in DPNH and TPNH levels 6 hours after oestrogen treatment. On the other hand, levels of some DPN- and TPN-dependent dehydrogenases are found to be increasing by 3 to 4 fold 72 hours after giving oestrogen (Bever et al, 1956; Mongkolkul & Grant, 1960; see also SECTION IV, Fig. IV. 1) while there is no evidence of any further increased rate of reoxidation of DPNH and TPNH. Moreover, "DPNH-oxidase" activity is reported to be rate limiting in the lactic acid dehydrogenase-"DPNH-oxidase" system (Bever et al, 1956). The rate of production of reduced coenzymes thus appears to be greater than their reoxidation rate, and an accumulation of the reduced coenzymes may be expected. It must be pointed out that the TPNH level, already higher than TPN in the uterine tissue (Table III. 2), is not likely to be increased drastically under the circumstances. DPNH level, however, may be raised substantially in view of the fact that this coenzyme exists mainly in the oxidised form (Table III. 2). These reasonings are well in accord with data obtained in the present investigation.

Although there is no dramatic change in the

concentrations of the pyridine nucleotides per unit weight of rat uterus following oestrogen administration, the absolute amount of the nucleotides per uterus does in fact increase, mainly by virtue of the increased weight of the organ. The rise in the amount of these pyridine nucleotides is probably accompanying the general uterine growth induced by the oestrogen administration. The rates of biosyntheses of pyridine nucleotides and nucleic acids are likely to depend on the rate of synthesis of adenine, one of the main components of these substances. The rates of incorporation of labelled substrates into adenine (and guanine) in the rat uterus is know to be increased very early after oestrogen administration (Mueller et al, 1958). However, the increases in the rates of biosyntheses of these bases do not promote the in vivo syntheses of pyridine nucleotides and the two types of nucleic acids (ribose nucleic acid, RNA ,and deoxyribose nucleic acid, DNA) at the same time. Thus the amount (mg/uterus) of RNA increases between 6 and 21 hours after oestrogen injection and that of DNA increases between 48 and 72 hours (Telfer, 1953). The increases in pyridine nucleotides, found in this investigation to parallel roughly uterine growth, probably start about 24 hours after oestrogen treatment, the period at which true growth begins (Mueller et al, 1958).

It is interesting that the sequences of the biosynthesis of RNA, DNA and pyridine nucleotides in the uterus, after oestrogen treatment agree well with observations on other aspects of uterine growth. Thus the early accumulation of RNA is probably necessary for an increase in protein biosynthesis, which also starts at an early stage after oestrogen treatment (Mueller et al, 1958); pyridine nucleotides appear to be formed at about the same time as other extra nuclear cell constituents. Finally, DNA does not accumulate until uterine cells are ready to divide.

Enhancement of the biosyntheses of nucleotides and nucleic acid required an increase in the supply of pentose phosphate. Glock (1955) and Glock & McLean (1958) suggest that in many tissues the pentose phosphate may come mainly from the hexosemonophosphate 'shunt' pathway of glucose oxidation. The evidence that there is an increase in the levels of 'shunt' enzymes in the rat uterus after oestrogen treatment will be found in SECTION IV of this thesis.

SECTION IV

EFFECTS OF OESTROGENS ON THE LEVELS OF PYRIDINE NUCLEOTIDE-LINKED DEHYDROGENASES IN THE RAT UTERUS.

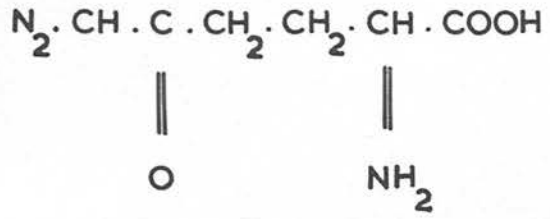
INTRODUCTION

In attempts to understand how hormones work in the body, many investigators have studied their effects on the levels of enzymes in various tissues. (Since it is possible that the hormones may influence the amount or the activity of a given enzyme or perhaps both, the term 'level' has been adopted and taken to cover any way in which the enzyme may be influenced by the hormone). It has been hoped that changes in the levels of certain enzymes, which may occur after the administration of a hormone, may bear some relationship to the physiological activity of the hormone, and thus throw some light on its mechanism of action. A list showing how some hormones, including oestrogens, affect the levels of many enzymes in animal tissues is given in the General Introduction (p. 10). The finding that DPNH and TPNH concentrations in the ovariectomised rat uteri increase 3 days after oestradiol administration (see SECTION III) prompted the study, about to be reported, of the effects of oestrogens on DPN- and TPN-dependent dehydrogenases in the rat uteri.

The three principal oestrogens found in man, oestradiol, oestrone and oestriol, are not known to be the natural oestrogens of the rat. Nevertheless they influence certain rat tissues in a manner similar to that found in man.

It may therefore be justifiable to compare their action on enzymes in the rat. It must also be borne in mind that the activity of these substances varies considerably with methods and techniques employed (see Merrill, 1958). It is thus interesting to study the relative activities of these three oestrogenic substances on the uterine levels of dehydrogenases to be investigated.

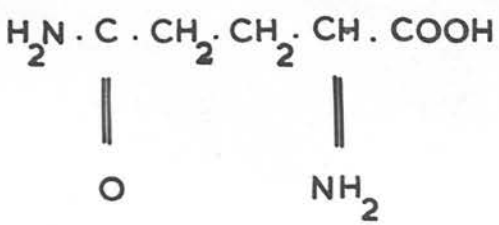
In an attempt to find whether any increases in dehydrogenase levels observed are due to increased enzyme activity or to synthesis of new enzyme protein, the effect of substances influencing protein synthesis or growth processes in general was studied. Progesterone, testosterone and DOC acetate when administered individually to ovariectomised rats in mg. quantities are reported to promote some uterine growth, but when each of these substances is administered with oestradiol to similar animals there are marked <u>decreases</u> in uterine growth as compared with the effect of oestradiol alone (Velardo,1959). MER-25 1(p-2-diethlaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol (see formula, p.18) has been shown to decrease the growth response to oestrogen in uteri of mice (Lerner <u>et al</u>, 1958). DON or 6-diazo-5-oxo-L-norleucine (formula shown on p. 71) has been shown to inhibit vaginal and uterine growth in mice given oestradiol (Paulson, 1960). Being similar in structure



6-Diazo-5-oxo-L-norleucine

(DON)

(c.f.



glutamine)

to glutamine, DON is known to compete with this compound in the synthesis of protein and of purines or RNA (see Buchanan, 1958), and RNA synthesis is now known to occur hand in hand with protein synthesis (see Zamecnik, 1958). DON probably inhibits uterine growth, induced by the administration of oestrogen, by interfering with protein synthesis directly and indirectly. The mechanism by which progesterone, testosterone, DOC acetate and MER-25 antagonise oestrogen effect is, however, not yet clear. The effect of *in vivo* administration, individually or together with oestradiol, of these steroids and oestrogen antagonists on the levels of uterine dehydrogenases has now been investigated.

Lastly, the effect of thyroxine on the levels of uterine dehydrogenases was studied. The thyroid hormone has been reported to increase rat liver levels of G6PDH and 6PGDH (Glock *et al*, 1956; Huggins & Yao, 1959), and it is of interest to know if it has any effect on similar enzymes in the uterus.

PLAN OF INVESTIGATIONS

In the experiments to be described in this Section, the levels of four pyridine nucleotide-linked dehydrogenases in the uterus of the rat were measured before and after modification of oestrogenic status of the animal. Injection of oestradiol was usually employed for this purpose but a comparative study of the effect of oestradiol, oestrone and oestriol on the rat uterine dehydrogenases was also made. The enzymes concerned are glucose-6-phosphate (G6PDH), 6-phosphogluconate (6PGDH), isocitric (ICDH), and lactic (LDH) dehydrogenases, located in the 'soluble' part of the cell sap. Bever et al (1956) have reported that LDH activity increases following oestrogen administration to ovariectomised animals. It was decided, however, to reinvestigate this effect using a simpler and more direct technique, in order to obtain results for comparison with data to be obtained on the study of TPN-dependent dehydrogenases.

The effects of in vivo administration of thyroxine and of antagonists of oestrogen action or of growth inhibitors referred to in the Introduction were also investigated.

A preliminary report of this work was presented

to the Biochemical Society (Mongkolkul & Grant, 1960).

EXPERIMENTAL

The Animals and Operations were similar to those described in SECTION III (p. 50).

Injections

The steroids for subcutaneous (s.c.) injection were dissolved in a minimum amount of ethanol and diluted with sesame or olive oil to the required concentrations. For intravenous injections, solutions of oestradiol in physiological saline were prepared by the method of Roberts & Szego (1947). Volumes of 0.1 ml were injected. Progesterone (1 mg), testosterone (1 mg) and DOC acetate (3 mg) were injected (s.c.) daily for three days. Unless otherwise stated, throughout this section, dosage refers to the amount administered each day. Injections (s.c.) were carried out on three consecutive mornings. The animals were killed 24 hours after the last injection.

A volume of 0.2 ml of a solution of 1 mg DON/ml physiological saline was injected five times intraperitoneally on the mornings and late afternoons of two consecutive days. The fifth injection was made on the morning of the third day. When oestradiol was to be given along with DON, 0.1 ml solution, containing 0.4 µg of the steroid, were injected subcutaneously four times, coinciding with the first four

injections of DON. This is a slight modification of the Paulson (1960) procedure.

The method of Lerner et al (1958) for the administration of MER-25 was followed with some modification. A suspension (17 mg/ml) of the compound in olive oil was made. Volumes of 0.1 ml were injected subcutaneously twice daily for 3 days.

In some experiments animals received a daily subcutaneous injection of 0.5 mg DL-thyroxine for 8 days as described by Glock & McLean (1955c). When oestradiol was given simultaneously, it was administered with the last 3 thyroxine injections.

Preparation of the uterine tissue and dry weight determination

This was done as described under Methods in SECTION III (p. 50).

Preparation of Enzymes

Pieces of uterine tissue, free from adhering fat and connective tissue and weighing about 100 mg, were homogenised for two minutes in a cone-shaped ground glass homogeniser containing about 10 ml of ice-cold solution of 0.15 M KCl. The volumes were measured and the homogenates centrifuged at 11,000 g. for 15 min. in an M.S.E. refrigerated

centrifuge. The supernatant solutions were not usually dialysed as described by Glock & McLean (1953) since this caused considerable loss in activity, and assays of enzymes were carried out on them without delay. However, when dialysis was employed, the extract was put in a cellophane sac and dialysed for 4 hours at 0° C against excess 0.15 M KCl solution. The KCl solution was changed after 2 hours. A magnetic stirrer was used to make the dialysis more efficient.

Determination of protein in the enzyme preparations was carried out by the Biuret method as follows. One ml of the uterine extract was mixed with 2 ml of 4 N-NaOH solution and 0.25 ml of a 1% solution of $CuSO4.5H_2O$, and the absorbance at 540 mμ was measured at exactly the 20th minute after the mixing. A standard solution of 1 mg/ml bovine serum albumin (Armour & Co.) was used with each determination. The amount of protein found in the extracts was approximately 1 mg/ml.

Enzyme assays and units

Spectrophotometric determinations were made with the Unicam SP500 spectrophotometer at room temperature (about 20°), using silica cuvettes of 1 cm. light path. The reduction or oxidation of pyridine nucleotides was

followed by measurement of change in optical density at 340 mμ. Only the initial velocity of the reaction was used in calculating enzyme activity. The molar extinction coefficient of reduced coenzymes was taken as 6,220. A unit of dehydrogenase activity is defined as that which either reduces or oxidises 1 μmole of the pyridine nucleotide per minute, and results are expressed as units per g. dry uterine weight. Similar values are obtained when results are expressed as enzyme units per mg. protein in the preparation.

The assay of G6PDH is a modification of the double substrate method of Glock & McLean (1953). The reaction mixture consists of 83 mM tris buffer pH 7.4; 17 mM $MgCl_2$; 0.17 mM TPN; 1.7 mM disodium-6-phosphogluconate; 3.3 mM disodium glucose-6-phosphate and 0.01-0.02 ml enzyme preparation, in a final volume of 0.3 ml. Micro-couvettes were used. Blanks are prepared without substrate.

The assay of 6PGDH is also based on that of Glock & McLean (1953). The reaction mixture contains exactly the same components as for the assay of G6PDH described above, except that G6P was omitted.

The assay of ICDH is carried out using the following reaction mixture: 83 mM tris buffer, pH 7.4; 3.3 mM $MnCl_2$; 2 mM DL-isocitrate, 0.17 mM TPN and 0.2 ml

of the uterine extract in a final volume of 3.0 ml. The blank couvette contains no TPN.

The assay of LDH is adapted from the method of Kubowitz & Ott (1943). The reaction mixture consists of 83 mM tris buffer, pH 7.4; 0.1 mM DPNH; 3 mM sodium pyruvate; and 0.05 ml of the enzyme preparation, in a final volume of 3.0 ml. The blank couvette contains no DPNH.

Reliability of methods

Four separate determinations of the dehydrogenase activities on a single uterus from an intact ('normal') rat were carried out. Results summarised in Table IV. 1 show that agreement within 10% SEM is obtained.

Table IV. 1.

Results from 4 separate determinations of dehydrogenase activities in a single rat uterus. Enzymic activities are expressed as means ± S.E.M.

Enzymes	Activities (units/g dry weight)	S.E.M. as % of means
G6PDH	18.3 ± 1.95	10.6
6PGDH	6.7 ± 0.27	4.0
ICDH	12.5 ± 0.33	2.6
LDH	123 ± 3.8	3.1

RESULTS

(i) Effect, on uterine dry weight and dehydrogenase levels, of prolonged oestrogen administration to ovariectomised rats.

Results summarised in Table IV. 2 show the dry weights and levels of G6PDH, 6PGDH, ICDH and LDH in uteri from 'normal' (see definition, p. 48), untreated ovariectomised, and ovariectomised rats injected subcutaneously with a daily dose of 10 µg of oestradiol for periods up to 24 days. The same results are shown in a diagrammatic form in Fig. IV. 1. The castrate rat levels of G6PDH and 6PGDH are only 37 and 34% of 'normal' rat values respectively. However, when oestradiol is administered to the ovariectomised animals, the levels of both enzymes increase markedly and steadily, reaching the 'normal' rat values by the 2nd day and maximum levels are attained on the 4th day after the start of oestrogen injections. The values of G6PDH and 6PGDH levels on the 4th day are 8 and 6 times those of castrate rat levels respectively. After attaining a maximum the levels of both enzymes are seen to decrease by the 5th and the 6th days, after which they are almost constant up to the 15th day. By the 24th day, however, the levels are slightly lower than on the 15th day. The significance of the second

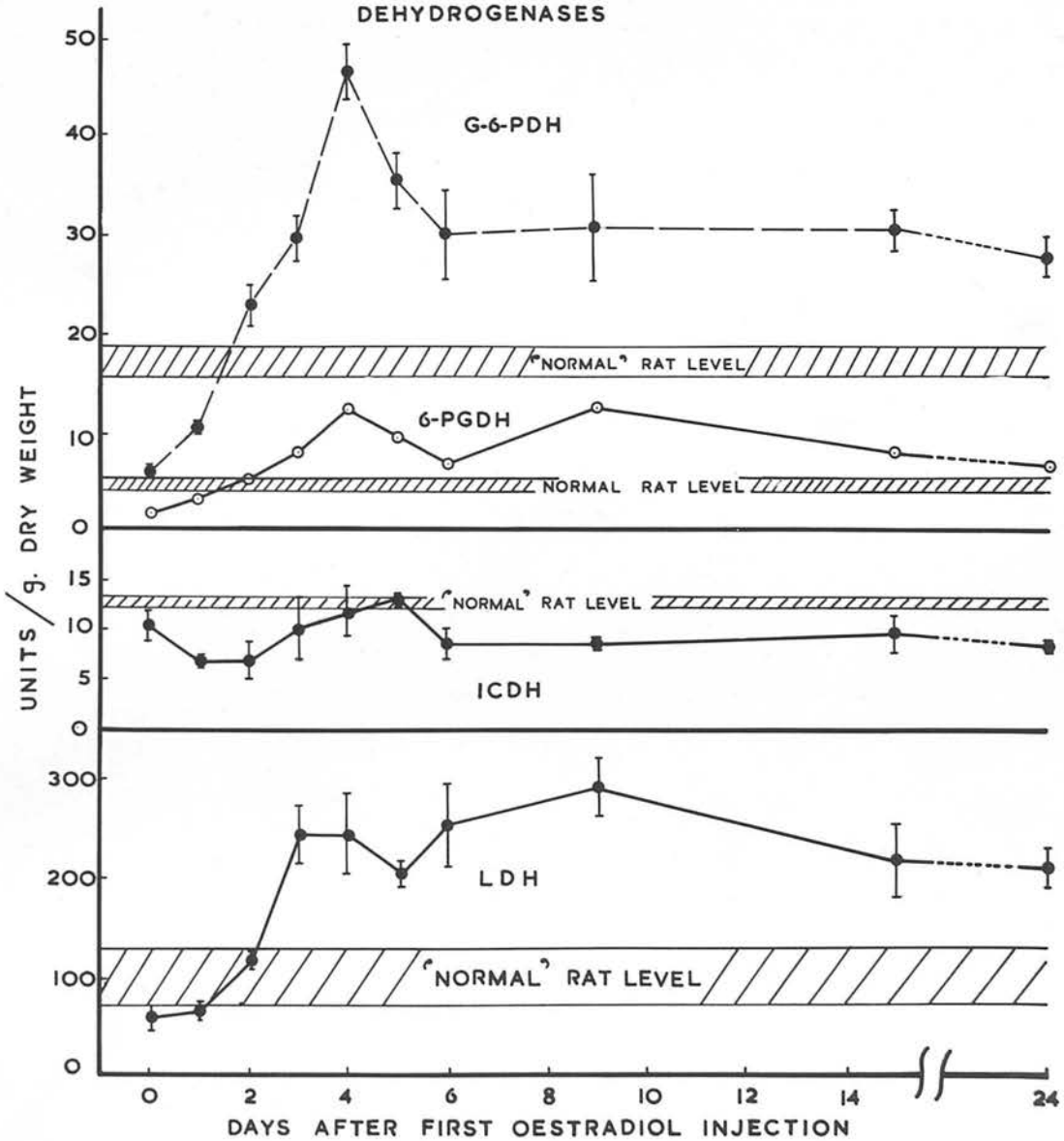
Table IV. 2.

Effects of prolonged injections of oestradiol (10 μg daily) to ovariectomised rats on the weight, and pyridine nucleotide-dependent dehydrogenases of the rat uterus (c.f. Fig. IV. 1). Enzymic activities are expressed as units per g. dry weight. Values shown are means with S.E.M.

Types of rats	Uterine dry-weight (mg.)	G6PDH	6PGDH	ICDH	LDH	No. of experiments
'normal'	62.1 ± 8.1	16.9 ± 1.2	4.7 ± 0.6	12.5 ± 0.5	102 ± 30	14
OVX*	15.6 ± 1.3	6.2 ± 0.5	2.0 ± 0.5	10.7 ± 1.3	58 ± 10	11
OVX + 10 μg OD** (s.c.) for 1 day	23.1 ± 1.1	10.4 ± 0.6	3.3 ± 0.5	7.2 ± 0.4	67 ± 3	3
OVX + 10 μg OD (s.c.) daily for 2 days	35.9 ± 5.5	23.1 ± 1.9	5.6 ± 0.3	7.3 ± 0.7	116 ± 3	3
OVX + 10 μg OD (s.c.) daily for 3 days	39.1 ± 2.2	30.1 ± 2.2	7.9 ± 0.1	9.9 ± 2.9	244 ± 23	6
OVX + 10 μg OD (s.c.) daily for 4 days	57.2 ± 3.6	46.6 ± 3.0	12.4 ± 0.1	11.4 ± 2.4	245 ± 33	5
OVX + 10 μg OD (s.c.) daily for 5 days	52.0 ± 5.2	35.6 ± 2.9	9.7 ± 0.3	13.1 ± 0.7	207 ± 9	3
OVX + 10 μg OD (s.c.) daily for 6 days	62.1 ± 6.3	30.4 ± 4.6	7.1 ± 0.3	8.8 ± 1.1	253 ± 45	3
OVX + 10 μg OD (s.c.) daily for 9 days	68.0 ± 7.8	31.0 ± 5.1	12.4 ± 0.2	8.5 ± 0.7	289 ± 31	3
OVX + 10 μg OD (s.c.) daily for 15 days	49.9 ± 1.6	30.9 ± 1.6	8.8 ± 3.0	9.5 ± 0.8	215 ± 37	3
OVX + 10 μg OD (s.c.) daily for 24 days	54.0 ± 2.3	27.7 ± 1.7	6.1 ± 0.5	7.9 ± 0.2	204 ± 20	3
OVX + 10 μg OD (i.v.) for 4 hrs.	17.1 ± 0.8	5.3 ± 0.3	1.8 ± 0.1	8.7 ± 0.4	74 ± 8	3
OVX + 10 μg OD (i.v.) for 24 hrs.	20.5 ± 1.2	10.3 ± 1.2	2.9 ± 0.3	10.7 ± 0.1	166 ± 5	2

* OVX = ovariectomised. ** OD = oestradiol.

FIG. (IV.1) A TIME-COURSE STUDY ON THE EFFECT OF OESTRADIOL ON LEVELS OF UTERINE DEHYDROGENASES



Note. For details, Fig. IV. 1 should be compared with Table IV. 2 (p 80). Vertical bars represent S.E.M. When no vertical bars are shown, S.E.M. are too small to be included in the diagram. Lines above and below striped areas labelled "'normal' rat level" represent means + S.E.M. and means - S.E.M. respectively.

peak of 6PGDH activity on the 9th day is not at present apparent. It may be noted that G6PDH level is always about 3 times higher than that of 6PGDH.

A different picture is obtained when the enzyme ICDH is considered. The level of this enzyme in the castrate animal is only slightly (15%) lower than in 'normal' rats. There is some insignificant fluctuation of this enzyme level after oestradiol administration. As a whole, the ICDH level remains slightly below the 'normal' rat value up to 24 days of the oestrogen treatment.

LDH level in the castrate rat uteri is only 57% of 'normal' rat value. After oestradiol administration, this enzyme level increases markedly, overtaking 'normal' rat level on the 2nd day and reaching maximum level on the 3rd day. This maximum level, about 4 times that of the castrate rat value, is retained with a slight tendency to decrease towards 'normal' rat on the 15th and 24th days after oestrogen treatment. It may be noted that LDH level is the highest of the enzymes investigated.

The uterine dry weight of the castrate rat is only 25% of 'normal' rat (Table IV. 2). The weight, however, increases gradually following the oestrogen administration returning to 'normal' rat level on the 4th day. This level is retained up to 24th day of the oestrogen

treatment. Because of this 4 fold increase in the uterine dry weight on the 4th day after oestrogen treatment, G6PDH, 6PGDH, ICDH and LDH levels at this time, expressed per uterus, are respectively 32, 24, 4 and 16 times the levels observed in the castrate rat uterus.

It is noted that the dry weight and levels of G6PDH, 6PGDH, ICDH and LDH are not significantly changed 4 hours after intravenous oestradiol administration to castrate rats (Table IV. 2). The changes in the dry weight and dehydrogenase levels in castrate rats 24 hours after intravenous injection of oestradiol are very similar to changes obtained after the same amount of the oestrogen is administered subcutaneously to these animals.

When mean values of dry weight and dehydrogenase levels per uterus of castrate rats, with and without oestrogen treatment, are expressed as percentages of similar values of 'normal' rats (Fig. IV. 2), it is possible to see how deviations from 'normal' occur following the stated treatment. Again the castrate rat uterine dry weight decreases to a quarter of the 'normal' value but increases steadily following oestradiol injections and reaches the 'normal' value about the 4th day. This 'normal' dry weight value is retained as long as oestrogen administration is continued. The 75% decrease in ICDH level as a result of ovariectomy, and its

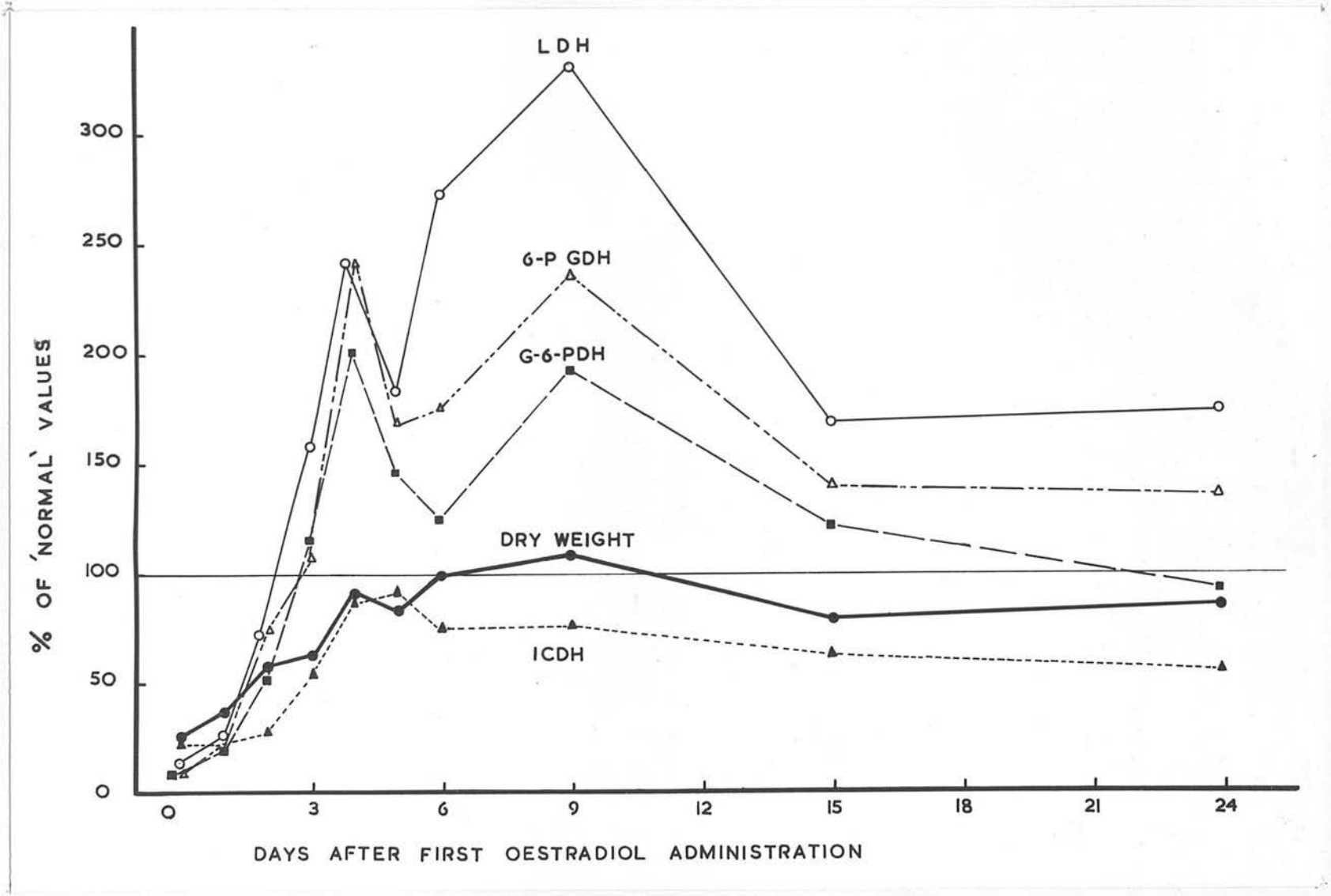


Fig. IV. 2. Rat uterine dry weight and dehydrogenase levels, as % of 'normal' values, at different stages of oestradiol administration (daily 10 μg subcutaneous injection).

gradual increase when oestradiol is subsequently injected, are closely similar to the changes in the dry weight. On the other hand, G6PDH, 6PGDH, LDH levels are more labile under the same circumstances. After ovariectomy they decrease to about 10% of 'normal' value, but start to increase sharply after oestrogen administration. The levels 4 days after oestradiol treatment are 200-250% of 'normal' values. The high level of the three dehydrogenases are retained, with some fluctuations, up to 9 days after the start of the treatment, but appear to decrease towards 'normal' values by the 15th and 24th days.

Effect of dosages of oestradiol:

Changes in the uterine dehydrogenase levels on subcutaneous administration of oestradiol to ovariectomised rats are influenced by the dosages of injected hormone. Fig. IV. 3 shows that the activities of G6PDH and 6PGDH increase linearly with doses of from 0.1 to 1.0 µg oestradiol, after which higher doses even up to 10 µg of the hormone promote only slight and insignificant increases in the enzyme activities. As little as 0.5 µg oestradiol appears to be enough to promote maximal response to the activity of LDH. ICDH activity is apparently not influenced by the steroid dosages.

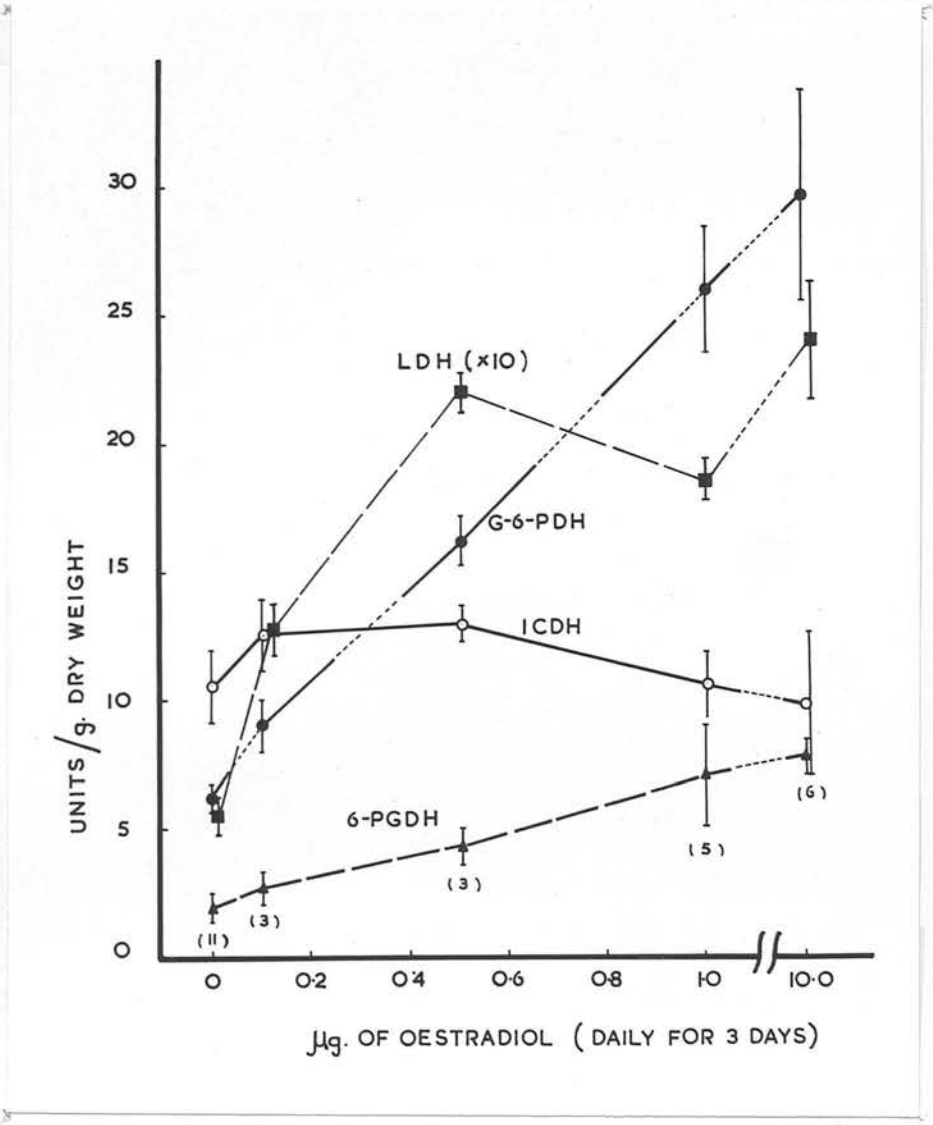


Fig. IV. 3. The relationship between uterine dehydrogenase activities and daily doses of oestradiol injected subcutaneously to castrate rats for three days. Numbers shown in parentheses indicate the number of experiments done at each oestrogen level. The vertical lines represent S.E.M.

Effect of different oestrogenic steroids:

Oestradiol, oestrone and oestriol appear to affect rat uterine levels of G6PDH, 6PGDH, ICDH and LDH in similar manner (Table IV. 3). However, the order of effectiveness in promoting changes in these dehydrogenases, especially of LDH, appears in general to be oestriol, oestrone and oestradiol, respectively.

(ii) Effect on the uterine dehydrogenases of oestrogen antagonists and thyroxine administered alone and together with oestradiol.

Results summarised in Table IV. 4 show that progesterone, testosterone and DOC acetate, when administered individually to the castrate rats, promote some uterine growth, accompanied by slight increases in the levels of G6PDH, 6PGDH and LDH. Progesterone and testosterone appear to be equally effective in promoting uterine growth, and DOC acetate the least effective of the three. When administered together with oestradiol, progesterone markedly inhibits the effects of oestradiol on the levels of the enzymes studied, especially those of G6PDH and LDH. Testosterone and DOC acetate also inhibit the oestradiol effect, but to a lesser extent.

Table IV. 4 also shows that either MER-25 or DON administered alone is in general without effect on the

Table IV. 3.

Effects of different oestrogenic hormones on the weight and dehydrogenases of the rat uterus. The stated amounts of oestrogen were administered (s.c.) daily to castrate rats for 3 days (see p. 75) Enzymic activities are expressed as units/g. dry weight. Values shown are means with S.E.M.

Types of rats	Uterine weight (mg)	G6PDH	6PGDH	ICDH	LDH	No. of experiments
OVX*	75 ± 6	6.2 ± 0.5	2.0 ± 0.5	10.7 ± 1.3	58 ± 10	11
OVX + 10 µg oestradiol	206 ± 4	30.1 ± 2.2	7.9 ± 0.1	9.9 ± 2.9	244 ± 23	6
OVX + 10 µg oestrone	233 ± 24	38.9 ± 7.3	9.1 ± 1.4	11.3 ± 0.6	381** ± 47	3
OVX + 10 µg oestriol	233 ± 19	48.1 ± 4.7	7.6 ± 1.4	9.5 ± 0.5	392** ± 25	3

* OVX = ovariectomised

** $p < 0.05$ c.f. OVX + 10 µg oestradiol.

Table IV. 4. Effects of oestrogen antagonists and thyroxine on the responses of dehydrogenases to oestradiol in the rat uterus. Injections were done as described on p. 75. Enzymic activities are expressed as units per g. dry weight. Values shown are means with S.E.M.

Substances injected to ovariectomised rats	Uterine wet weight (mg.)	G6PDH	6PGDH	LDH	No. of experiments
Nil - control	75 ± 6	6.2 ± 0.5	2.0 ± 0.5	58 ± 10	11
OD*	200 ± 17	26.3 ± 2.6	7.2 ± 1.6	188 ± 8	5
progesterone	144[a] ± 26	8.9 ± 1.0	2.7 ± 0.5	60 ± 4	3
OD + progesterone	168 ± 25	15.3[b] ± 0.1	3.8 ± 0.1	104[b] ± 6	3
DOC acetate	99 ± 8	7.8 ± 1.1	1.8 ± 0.4	81 ± 19	3
OD + DOC acetate	194 ± 51	19.7 ± 1.6	4.2 ± 0.2	201 ± 5	3
Testosterone	153 ± 8	8.3 ± 0.3	3.1 ± 0.3	66 ± 10	2
OD + testosterone	175 ± 15	20.3 ± 2.3	5.1 ± 0.2	122 ± 11	2
MER-25	102 ± 4	5.9 ± 0.3	1.5 ± 0.2	73 ± 4	3
OD + MER-25	154 ± 8	11.2[b] ± 1.5	2.1 ± 0.2	131[b] ± 19	3
OD'**	155 ± 15	22.6 ± 1.0	5.8 ± 0.3	191 ± 31	4
DON (1 mg)	76 ± 4	6.8 ± 0.2	2.3 ± 0.2	136 ± 7	3
OD' + DON (1 mg)	86[c] ± 4	14.8[c] ± 2.0	2.9[c] ± 0.2	122 ± 31	3
OD' + DON (2 mg)	87	11.0	2.6	124	1
thyroxine	103 ± 9	6.9 ± 0.2	1.1 ± 0.1	60 ± 6	3
OD + thyroxine	285 ± 19	26.1 ± 2.3	3.8 ± 0.4	169 ± 24	3

* OD = oestradiol (1 µg daily) ** OD' = oestradiol (1.6 µg over 2 days, see p.75).

[a] $0.05 > p$ c.f. OVX control [b] $0.05 > p$ c.f. OD [c] $0.05 > p$ c.f. OD'

levels of G6PDH, 6PGDH and LDH. However, when either compound is injected along with oestradiol, there are pronounced decreases in the uterine weight and dehydrogenases levels.

Thyroxine administered to castrate rats has no effect on the uterine levels of G6PDH, 6PGDH and LDH. Moreover, the thyroid hormone administered together with oestradiol, shows neither synergistic nor antagonistic effects as far as the levels of the three dehydrogenases in the rat uterus are concerned.

DISCUSSION

As predicted in SECTION III, there are marked increases in levels of DPN- and TPN-dependent dehydrogenases in the uteri of ovariectomised rats treated with oestrogen. However, not every enzyme shows this upward response. Thus G6PDH and 6PGDH show an increase but the level of ICDH is uninfluenced. The effects observed on G6PDH and 6PGDH are in agreement with the report of Scott & Lisi (1960) who find a 3 fold increase in the levels of these enzymes in the endometrium and 2 fold increase in the myometrium of the ovariectomised rat uteri, 36 hours after an intraperitoneal injection of 100 µg oestradiol. Both enzyme levels are reported by the same authors to fall significantly by the 76th hour, presumably due to the destruction in the rat body of the administered oestrogen. It is seen, however, from the results of the present investigation (Fig. IV. 1) that repeated administrations of smaller dosages of the oestrogen (10 µg per day) produce much higher increases in the levels of the two enzymes by the 4th day after the first injection. It may be noted that the degree of enzymic response is dependent on the amount of oestrogen administered. A minimum dosage of 0.1 µg per day appears to be necessary to produce a significant increase in the dehydrogenase levels, and 1 µg per day is the dosage above which no

further significant increase is obtained (Fig. IV. 3). The method of oestrogen administration does not appear to have any influence on the enzymic levels, as judged from observations made up to 24 hours after a single subcutaneous, intravenous or intraperitoneal injection of oestrogen (Table IV. 2 and data from Scott & Lisi, 1960).

The observation of Bever et al (1956) that oestriol is more active than oestradiol or oestrone in increasing the LDH level in the rat uterus, has been confirmed and the same observation has now been made for G6PDH (Table IV. 3) but is not found with 6PGDH.

Huggins & Yao (1959) report that the levels of 6PGDH in the liver increase 2 fold, 10-13 days after oestradiol treatment but the G6PDH level does not change in the same period. This observation was confirmed by the author in the preliminary experiments, but there is no sign of a similar phenomenon with uterine 6PGDH or G6PDH when animals were subjected to the same oestrogen treatment for up to 24 days. This finding may be cited as an example of a hormone having different effects on different tissues. Another similar example is that of thyroxine which increases both the G6PDH and 6PGDH levels in the liver (Glock et al, 1956; Huggins & Yao, 1959) but is without any effect on the uterine enzymes (Table IV. 4).

One outstanding effect of oestrogen administration to the ovariectomised rat is its 'normalising' on the uterus, i.e. the tendency to restore conditions (e.g. dryweight and enzymic activities) to their pre-ovariectomy values. There are however considerable differences in the ways in which the different enzymes approach the 'normal' values. The increase of ICDH level after oestradiol treatment is slow, and parallel to the rise in the uterine dryweight. Both reach the 'normal' levels 4 days after the start of oestrogen administration (Fig. IV. 2), and both ICDH level and uterine dryweight remain 'normal' if the administration of oestrogen is continued. This suggests that the increase in ICDH level may be due to synthesis of specific protein having ICDH activity at about the same pace as general growth. On the other hand, the levels of G6PDH, 6PGDH and LDH rise more abruptly and more extensively after the oestrogen administration, and pass 'normal' levels 2 days after oestrogen is first administered. The maximum values attained are 2-3 times those found in 'normal' rats. These high levels are maintained for a period of up to 9 days before finally decreasing towards 'normal' values even with continued daily injections of oestradiol. It thus appears possible that the specific proteins having these three dehydrogenase activities are being synthesised at a

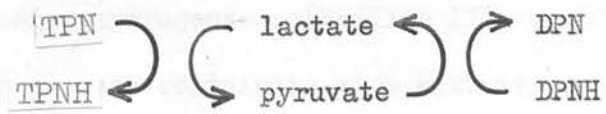
much faster rate than the general growth. However, the ultimate results are the same as before, viz. that 'normal' values are attained and maintained as long as the supply of oestrogen is continued.

Judging from the results obtained, there appears to be a close relationship between the increases in the levels of uterine dehydrogenases studied and the uterine growth, as measured by the increase in the uterine dryweight. The highest levels of G6PDH, 6PGDH and LDH are obtained when growth is also at its maximum, i.e. 4 days after oestrogen treatment commences. There is only a small increase in the weight of uteri from castrate rats when they are killed, either (a) 1 day after a single injection of 10 µg oestradiol, (b) 3 days after daily injections of 0.1 µg oestradiol or (c) 3 days after daily injection of 1 mg of progesterone, testosterone or DOC acetate. Under such conditions the increases in the levels of the three dehydrogenases are also slight. Furthermore, when the uterine growth is restricted by the administration, together with oestradiol, of substances inhibiting growth or protein synthesis, viz. MER-25, DON, and progesterone, testosterone and DOC acetate, there are correspondingly decreased effects of oestradiol on the levels of enzymes studied. It may be noted that these substances which are potent oestrogen antagonists, such as MER-25, DON

or progesterone, are more successful in this respect than weak antagonists like testosterone or DOC acetate. It is also interesting that thyroxine, which neither promotes nor inhibits uterine growth induced by oestrogen, has no effect on levels of enzymes investigated. The fact that increases in enzymic levels and uterine growth always occur together appears to support the theory that the increases observed after oestrogen treatment are a result of formation of new enzyme protein, rather than a direct oestrogen stimulation of existing enzyme molecules. The view that oestrogen does not directly activate dehydrogenase molecules is strengthened by the failure to demonstrate an increase in the activity of the enzymes under consideration, by oestradiol added *in vitro* (Bever, 1958; Scott & Lisi, 1960).

The physiological significance of the increases in dehydrogenase concentrations observed after oestrogen administration is difficult to assess. It is only reasonable to suppose that enzymes, the levels of which do not change markedly on ovariectomy and subsequent oestrogen administration, are those primarily concerned with normal day to day metabolism, and are not specifically involved in growth processes induced by oestrogen administration. ICDH belongs to this group of enzymes. On the other hand, other uterine enzymes such as G6PDH, 6PGDH and LDH, whose

concentrations fall and rise extensively according to the oestrogenic status of the animals, are also likely to take part in the processes which bring about the characteristic uterine growth in response to oestrogen. The possible significance of the LDH-DPNH-oxidase system in controlling the reversible oxidation and reduction of lactate and pyruvate at the end of glycolytic sequence of reactions has already been mentioned by Bever (1958). As far as growth is concerned, LDH could provide DPNH which can then be oxidised via the cytochrome system giving ATP, i.e. increasing the free energy supply for endergonic biosynthetic processes. This view is supported by the fact that the DPNH concentration in rat uteri 3 days after oestrogen treatment (when level of LDH is at its highest) is also found to be markedly increased (see SECTION III, p. 61). Another aspect of the elevated LDH level which may be of physiological importance is its possible role as a promoter of transhydrogenation. LDH, which can react with either DPN or TPN, is known to mediate in the transfer of hydrogen (Navazio, Ernster & Ernster, 1957; Holzer & Schneider, 1958) according to the following scheme:-



In this way LDH is able to regulate metabolic processes by controlling the flow of electrons, a function attributed to transhydrogenase already mentioned in SECTION I (p. 7). However, the quantitative significance of this LDH function in the uterine cells is still not certain in view of the criticism of Stein, Kaplan & Ciotti (1959) that only a small amount of transhydrogenation is mediated by LDH. The fact that DPN/DPNH and TPN/TPNH ratios, in the uterine tissue after oestrogen treatment, do not change correspondingly and in opposite directions diminishes the possibility that transhydrogenation is taking place to a significant extent, either by the action of LDH or by transhydrogenase (see SECTION III, p. 64).

The physiological significance of G6PDH and 6PGDH for growth processes could possibly be related to their dependence on TPN. Their functioning is responsible for the production of reduced TPN which appears to be a specific reductant for many biosynthetic processes (Horecker & Hiatt, 1958; Dickens et al, 1959). A slight increase in TPNH concentration in rat uteri obtained after oestrogen treatment has been discussed in relation to uterine levels of TPN-linked dehydrogenases (SECTION III, p. 66). In addition in tissues containing high activity of the hexosemonophosphate ('shunt') enzymes, pentose phosphate,

required in the biosynthesis of nucleotides and nucleic acid could come from the direct oxidative decarboxylation of glucose-6-phosphate (Glock & McLean, 1958). It thus appears logical that there should be only minimal activities of G6PDH and 6PGDH in the atrophic castrate rat uteri, and that their levels should be increased 6 to 8 fold following growth induced by the administration of oestrogen. It is interesting that the levels of G6PDH and 6PGDH in uteri of 'normal' rats or castrate rats 4 days after oestrogen treatment, although they are much lower than those in the mammary gland at the height of lactation, are generally higher than those in adrenal or liver (Table IV. 5). On the other hand, similar enzymic levels in the castrate rat uteri are low, and almost in the same range of the levels in the non-lactating mammary gland. High levels of G6PDH and 6PGDH are known to be associated with tissues involved in active biosynthesis. For example, Glock & McLean (1958) report a 40 fold increase in G6PDH and 10 fold increase in 6PGDH activities in the rat mammary glands from the start of pregnancy to the height of lactation. These increases are concluded to be associated with milk production. The high activities in the adrenal glands of the two enzymes just mentioned (Glock & McLean, 1954; Kelly, Nielson, Johnson & Vestling, 1955) may well be linked with the production of

Table IV. 5.

Activities of G6PDH and 6PGDH in the rat tissues expressed as units per g. wet weight with S.E.M. (1 unit is equivalent to 0.01 μ moles TPNH production per min.)

Tissues	G6PDH	6PGDH (pH 7.4 or 7.6)	References
adrenal gland	163 ± 25	185 ± 9	Glock & McLean (1954)
liver (female)	104* ± 12	130* ± 12	Glock & McLean (1954)
liver (male)	46 ± 3	59 ± 8	Glock & McLean (1954)
skeletal muscle	8 ± 1	8 ± 0.3	Glock & McLean (1954)
mammary gland (20 days pregnant)	86	50	Glock & McLean (1954)
mammary gland (21 days lactation)	5452	883	Glock & McLean (1954)
uterus (castrate)	128 ± 9	42 ± 4	This investigation
uterus ('normal')	304 ± 24	90 ± 11	This investigation
uterus (castrate with 4 days oestrogen treatment)	850 ± 52	229 ± 22	This investigation

* Approximately the same results were also obtained in the preliminary experiment in the present investigation.

TPNH required for adrenocortical steroid biosynthesis (Grant & Brownie, 1955). When adrenocorticotrophic hormone (ACTH) is administered to human subjects, causing adrenal growth and increases in steroid production, an increase in G6PDH and 6PGDH activity has been demonstrated (Studzinski, Symington & Grant, 1960). In view of these considerations it seems reasonable to assume that the increase in levels of G6PDH and 6PGDH in the uterus in response to administered oestrogen is to provide an environment favourable to biosynthetic processes associated with growth.

It must be pointed out that the assays of enzymic levels in this investigation, are carried out under optimal conditions, i.e. in the presence of excess of substrates and coenzymes. In the rat uterus concentrations of di- and triphosphopyridine nucleotides are shown here to be low (see SECTION III) and endogenous substrates having TPN linked dehydrogenases are also found to be low (Scott & Lisi, 1960). Racker (1954) suggests that in steady states in cells, concentrations of intermediates (substrates) are well below those required to saturate the enzymes. Conditions are therefore such that enzymes, using the same substrate, are competing for substrate rather than the reverse. A large increase in the activity of G6PDH thus greatly enhances the ability of the enzyme to compete for its substrate,

glucose-6-phosphate. This may result in a shifting of the oxidation of glucose from glycolysis towards hexose monophosphate 'shunt' pathway. The pathway of glucose oxidation followed in the uterus is therefore investigated and reported in the next Section.

SECTION V

THE EFFECT OF OESTRADIOL ON THE PATHWAYS OF GLUCOSE CATABOLISM IN THE RAT UTERUS.

INTRODUCTION

Glycolysis or the Embden-Myerhof pathway is indisputably the main route of carbohydrate metabolism in most animal tissues, and the major alternative pathway appears to be via the pentose phosphate or hexosemonophosphate 'shunt' (Racker, 1954; Gunsalus et al, 1955; Dickens, 1955 and 1958; Horecker & Hiatt, 1958; Dickens, Glock & McLean, 1959). However the 'shunt' pathway has been shown to be actively responsible for glucose catabolism in many tissues including the lactating mammary gland (Abraham et al, 1954; Glock et al, 1956a), liver (see Dickens et al, 1959), corneal epithelium (Kinoshita, Masurat & Helfant, 1956), leucocytes (Coxon & Robinson, 1956), bone marrow (Bloom, 1955) and most endocrine tissues (see Field et al, 1960). A general view at present appears to be that the 'shunt' pathway is more closely related to biosynthetic rather than to energy yielding processes (see p. 12).

Oestrogen is known to influence glucose metabolism. Thus it is reported that uteri from ovariectomised rats up to 24 hours after oestradiol administration show increases in glucose utilisation, lactate accumulation, and oxygen consumption compared with untreated ovariectomised animals (Roberts & Szego, 1953). These results are interpreted by these workers as a stimulation

of glycolysis by oestrogen. However, it was also found in the same investigations that not all glucose disappearance can be accounted for by lactate accumulation. For instance, when glucose utilisation is still increasing in uteri, 24 hours after oestradiol injection, there is a marked decrease in the level of lactate produced. This may be an indication that the extra glucose is being metabolised either more completely, as suggested by Roberts & Szego (1953), or by some pathway other than glycolysis. The marked increases in the levels of G6PDH and 6PGDH in the uteri of castrated rats after oestrogen treatment (see SECTION IV, Fig. IV. 1) also indicate that the 'shunt' pathway of glucose metabolism in the uterus may be affected by oestrogen. It is therefore of interest to investigate the 'shunt' activity in the uterus from rats of different oestrogenic status. The knowledge of whether oestrogens regulate the two pathways of glucose metabolism may throw some light on the mechanism of action of these hormones. Recently McLeod & Hollander (1961) in a preliminary report claim that there is no increase in the 'shunt' activity of the castrate rat uterus 24 hours after oestradiol injection, compared with castrate rat control. However, this observation is not surprising in view of the low levels of G6PDH and 6PGDH in the castrate rats 24 hours after

oestradiol administration as reported in SECTION IV (Fig. IV. 1). It is therefore still plausible that the 'shunt' pathway may be more active in the uterine tissue where levels of G6PDH and 6PGDH are high such as found in castrate rats, 3-4 days after oestrogen administration.

It has been realised recently that the re-oxidation of TPNH is a factor controlling the activity of the 'shunt' (Kinoshita, 1957; Cahill, Hastings, Ashmore & Zottu, 1958; Brin & Yonemoto, 1958; McLean, 1959). These workers show that marked stimulation of 'shunt' activity can be obtained by the addition, to the incubation mixture, of (i) substances such as methylene blue or phenazine methosulphate, which oxidise TPNH to TPN directly (Cahill et al, 1958; Brin & Yonemoto, 1958; McLean, 1959); (ii) substances which promote TPNH utilisation such as insulin, which increases the rate of fatty acid biosynthesis (Abraham, Cady & Chaikoff, 1957; McLean, 1959), in the process of which TPNH is oxidised to TPN (Hele, 1958); (iii) substances which promote transhydrogenation and presumably re-oxidise TPNH, e.g. pyruvate (Kinoshita, 1957; see also p. 91). It is therefore interesting to investigate if the 'shunt' activity in the rat uterus can be stimulated by these substances.

The hexosemonophosphate shunt is known to

proceed unaffected by iodoacetate, iodoacetamide or fluoride ion which inhibit glycolysis completely (Dickens & Glock, 1951). The extent of glucose utilisation in the rat uterus in the presence of any of these inhibitors may indicate the relative activity of the 'shunt' in this tissue compared with glycolysis. The results obtained may be compared with those from experiments with isotopically labelled glucose.

Various methods have been devised for determining the relative importance of the glycolytic and 'shunt' pathways in the catabolism of glucose by tissues. One method involves the use of glucose specifically labelled with ^{14}C in position 1 or 6. The theoretical basis of this method is that in glycolysis, glucose molecules yield two similar C_3 fragments and therefore both the C-1 and C-6 of glucose molecules suffer the same fate. They are equally oxidised to CO_2 via the Kreb's citric acid cycle. On the other hand, in the hexosemonophosphate 'shunt', the C-1 of glucose is removed as CO_2 in the first three enzymic steps while the C-6 will be oxidised to CO_2 only after recycling. C-1 of glucose is therefore oxidised to CO_2 at a faster rate than C-6, assuming a slow recycling process, which is in fact found in most tissues. It thus appears that the ratio of $^{14}CO_2$ liberated from the oxidation

of Glucose-1-^{14}C and Glucose-6-^{14}C (called the C-1/C-6 quotient) is 1 if glycolysis is the sole catabolic pathway. Ratios higher than 1 are usually interpreted as an indication that the 'shunt' pathway is operating in the tissues concerned. Due to the simplicity and convenience of this method it is most widely used in comparing the significance of the two pathways of glucose catabolism in many tissues. Other methods which have been employed are much more complicated and difficult to carry out. These methods involve the measurement of the incorporation of ^{14}C (from specifically labelled glucose) into lactate, fatty acids, acetoacetate, etc., and the relative contribution of the glycolytic and 'shunt' pathways is calculated in a rather complicated fashion. It must be pointed out that the precise quantitative evaluations of results obtained by most methods employed so far are difficult and open to criticism due to the uncertainty of assumptions made in the calculations (Wood, 1955). At best, the results obtained can be considered as an indication of the relative activities of glycolytic and 'shunt' pathways.

PLAN OF INVESTIGATIONS

The present investigation was intended to be of a preliminary nature only, and no attempt was made to evaluate precise quantitative significance of the two pathways of glucose catabolism already mentioned.

Uteri from (a) intact rats (i.e. 'normal' rats, see definition, p. 48), (b) ovariectomised rats untreated, and (c) ovariectomised rats 3-4 days after oestradiol administration, were incubated with either glucose-1-^{14}C or glucose-6-^{14}C. CO_2 evolved from the incubation was trapped in alkali and precipitated as $Ba^{14}CO_3$, the radioactivity of which was measured. It was thus possible to compare the C-1/C-6 quotients obtained with uteri from animals of different oestrogenic status. The effects of phenazine methosulphate, insulin and iodoacetamide on the oxidation of C-1 and C-6 of glucose in these three different types of uterine tissues, (a), (b) and (c), have also been studied.

The rates of glucose utilisation and O_2 consumption of uteri of rats (b) and (c) described above were compared with those of Robert & Szego (1953) who report marked increases in these rates up to 24 hours after a single administration of oestradiol.

EXPERIMENTAL

The Animals and Operations were similar to those described in SECTION III (p. 50).

Oestradiol injections

0.1 ml of a solution containing 10 µg oestradiol in sesame oil was injected subcutaneously into the castrated rats. The injections were repeated daily for 3-4 days as stated in the text.

Preparation of chopped tissue

Rat uteri were removed, cleaned and small portions were reserved for dry weight determination as described in SECTION III (p. 50). The uteri were then placed on a small cooled plastic disc sitting on the chilled metal platform of a McIlwain Tissue Chopper (H. Mickle, Gomshall, Surrey; see McIlwain, 1961) and chopped transversely with the slice thickness setting adjusted to 0.25 mm. Usually chopped tissue from a number of uteri were pooled together and 100-200 mg. portions were weighed into each incubation vessel.

Incubation with radioactive glucose

Glucose-1-^{14}C and glucose-6-^{14}C were obtained

from the Radiochemical Centre, Amersham. They were diluted with carrier glucose to give a specific activity of 50 μc per mmole. A solution containing 100 μ moles (5 μc) glucose per ml was prepared. Of this solution 0.05 ml volumes were delivered, by means of automatic pipettes, into a series of boiling tubes (1" X 4.5") containing chopped uterine tissue suspended in 2.5 ml of freshly made Krebs-Ringer bicarbonate solution (Umbreit, Burris & Stauffer, 1957). Other reagents (0.1 ml of stated quantities of hormones, dyes, etc.), when indicated, were added at this stage.

After gassing each tube for 1 minute with O_2:CO_2 (95:5) mixture, all tubes were closed tightly with rubber bungs, fitted with two glass tubes, as shown at A and B in Fig. V. 1. Both the stopcock at A and the screw clip at B were kept tightly closed. The tubes were then incubated at 37° with gentle shaking for 1-3 hours. At the end of the incubation, they were removed and rubber connections at B were attached to 'bubbling tubes' containing 5 ml of freshly prepared (CO_2 free) 3N. NaOH and the screw clip at B opened. One ml portions of 6N. H_2SO_4 were delivered via A to stop the reaction. A very gentle stream of N_2 was then allowed to sweep through the incubation mixture (via A) for at least an hour to ensure a complete absorption of CO_2 in the 'bubbling tube.' It was found

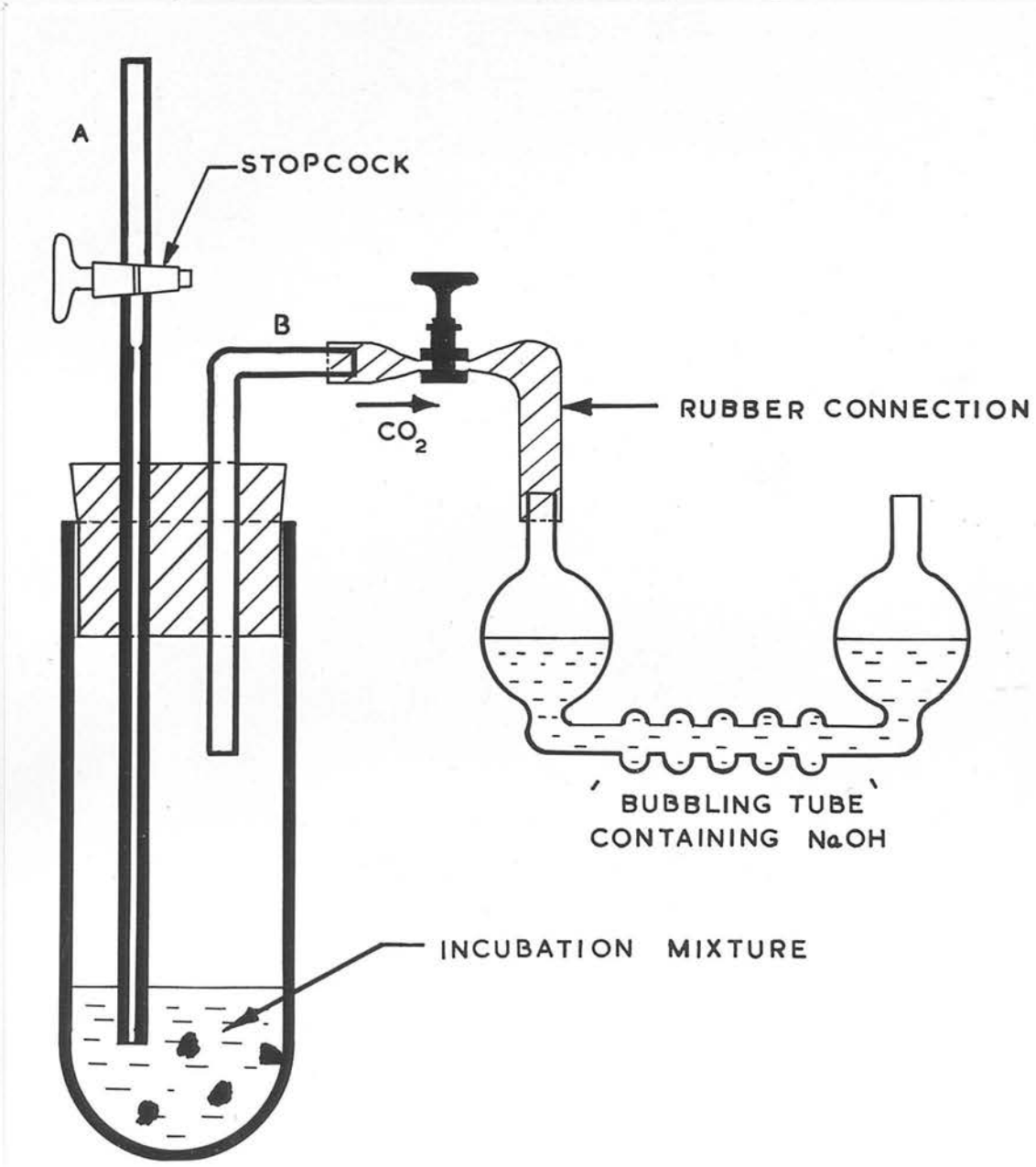


Fig. V. 1. Apparatus for incubating chopped uterine tissue and the 'bubbling tube' containing alkali for trapping liberated CO_2 (see p103).

in a preliminary experiment that when the first 'bubbling tube' was replaced after 1 hr. by a second tube, and the N_2 stream continued for a further hour, there was no apparent radioactivity trapped in the second tube indicating the completeness of absorption of CO_2 by the first tube.

When the bubbling procedure was completed, the alkaline solution containing $^{14}CO_2$ in the 'bubbling tube' was transferred quantitatively into 10 ml graduated flasks and made up to the mark with CO_2-free distilled water. The solutions were then ready for precipitation with $BaCl_2$.

Precipitation

The techniques described in 'Handbook of Isotope Tracer Method' (ed. W. Sakami) were followed.

The apparatus shown in Fig. V. 2 was used in the precipitation of the absorbed $^{14}CO_2$ as $Ba^{14}CO_3$. Two ml of 0.2 M $BaCl_2$ solution (CO_2 free) was introduced into the stainless steel tube sitting tightly on a weighed filter paper which, in turn, was sitting on a flat sintered glass filter fitted to a flask with connection to a water pump. If suction is not applied this solution does not pass through the filter. Four ml (in duplicate) of the alkaline solution containing $^{14}CO_2$ were then added slowly and with stirring (continued for 1 minute). After the addition was completed,

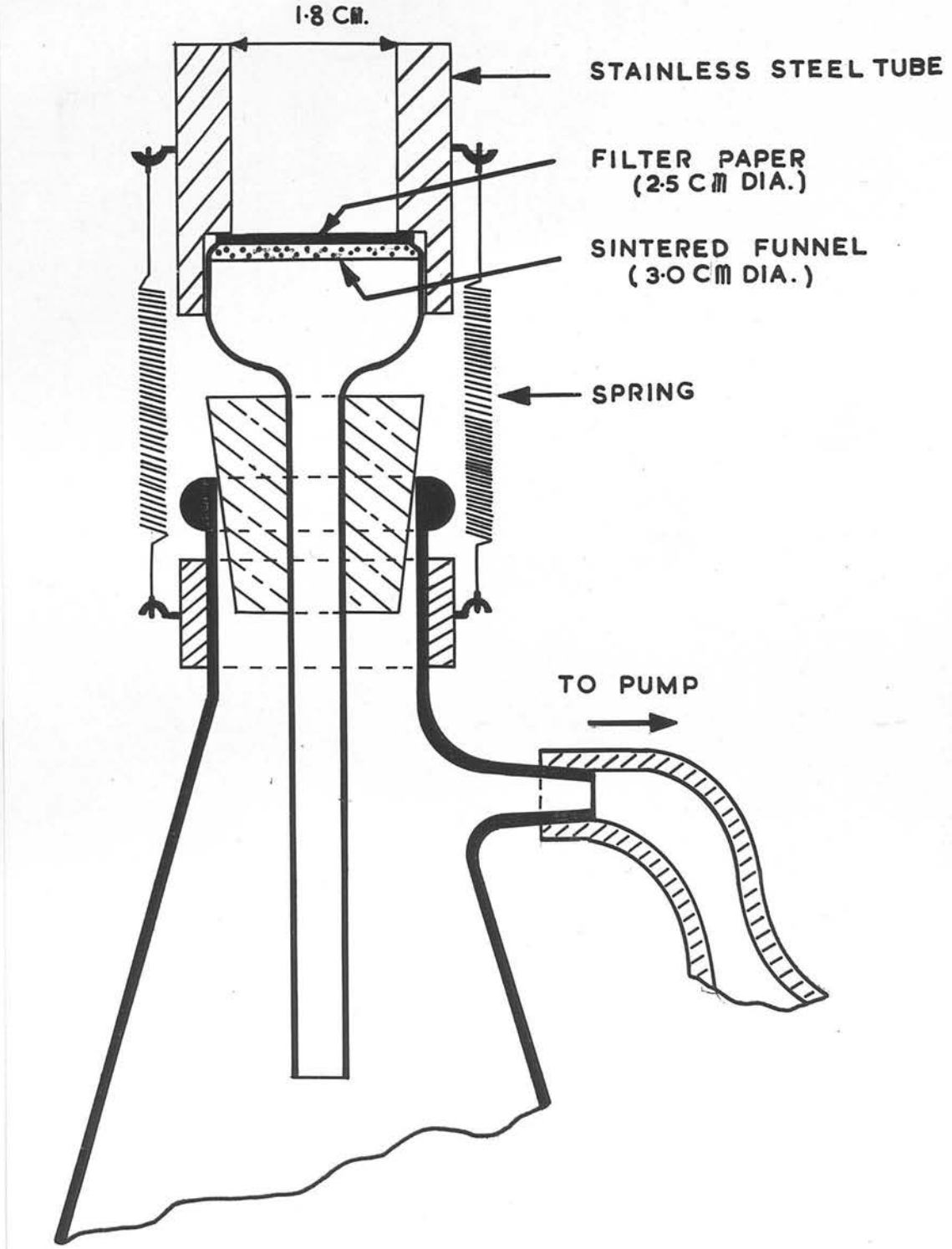


Fig. V. 2. Apparatus used for the precipitation of $Na_2^{14}CO_3$ as $Ba^{14}CO_3$
(see p 104).

a further 1 minute was allowed before the pump was started. The $Ba^{14}CO_3$ precipitate collected on the filter paper was well washed with CO_2 free-distilled water, and sucked dry by the pump before removal and drying under an "Angle Poise" lamp with a 60 watt bulb at a distance of 30 cm. A heavy metallic ring was carefully placed on the rim of the filter paper containing precipitates to prevent curling up. When dry the weights of the precipitates were determined so that corrections for radioactivity self-absorption could be made on individual precipitates. The area of the precipitate on the filter paper disc was 2.5 $cm.^2$ and precipitates of about 4 mg/cm^2 were normally obtained. The filter papers containing radioactive $BaCO_3$ precipitate were stuck on to plastic planchets with the help of a glue (Durofix). When dry, the preparations were ready for radioactivity measurement.

Radioactivity measurements

Radioactivity was measured using the 'micromil' window of an automatic counter (Nuclear, Chicago).

A self-absorption curve for the counter was made by plotting the radioactivity measurements of a series of $Ba^{14}CO_3$ precipitates, obtained by precipitation of $BaCl_2$ with $Na_2{}^{14}CO_3$ solutions (Radiochemical Centre, Amersham)

containing the same amount of radioactivity but an increasing amount of Na_2CO_3 carrier, against their "thickness" (mg/cm^2). Fig. V. 3 shows that the 'infinite thickness' value for the counter employed is more than 50 mg/cm^2 which is rather high. It was thus decided to count $Ba^{14}CO_3$ precipitates of thin thickness (3-5 mg/cm^2), so that the high dilution of the radioactivity and the difficulty in making 'infinite thickness' preparations would be avoided. Three separate self-absorption curves (covering thicknesses of 1 to 5 mg/cm^2) were prepared as described above, and extensions of the graphs to 'zero thickness' were made (Fig. V. 4, tophalf). Taking 'zero thickness' readings as 100% activities, (i.e. no self-absorption), 'correction graphs' for self-absorption were then made by plotting % radioactivity against 'thickness' of the precipitates (Fig. V. 4, lower half). The mean value of the slopes of the three 'correction graphs' was used as a standard graph for correcting all radioactivities of $BaCO_3$ preparations to activities at 'zero thickness' (usually expressed in a final form as counts per min. (cpm) per mg. dry uterine weight).

Oxygen consumption determination

Oxygen consumption was determined by the usual Warburg technique (Umbreit et al, 1957). The main

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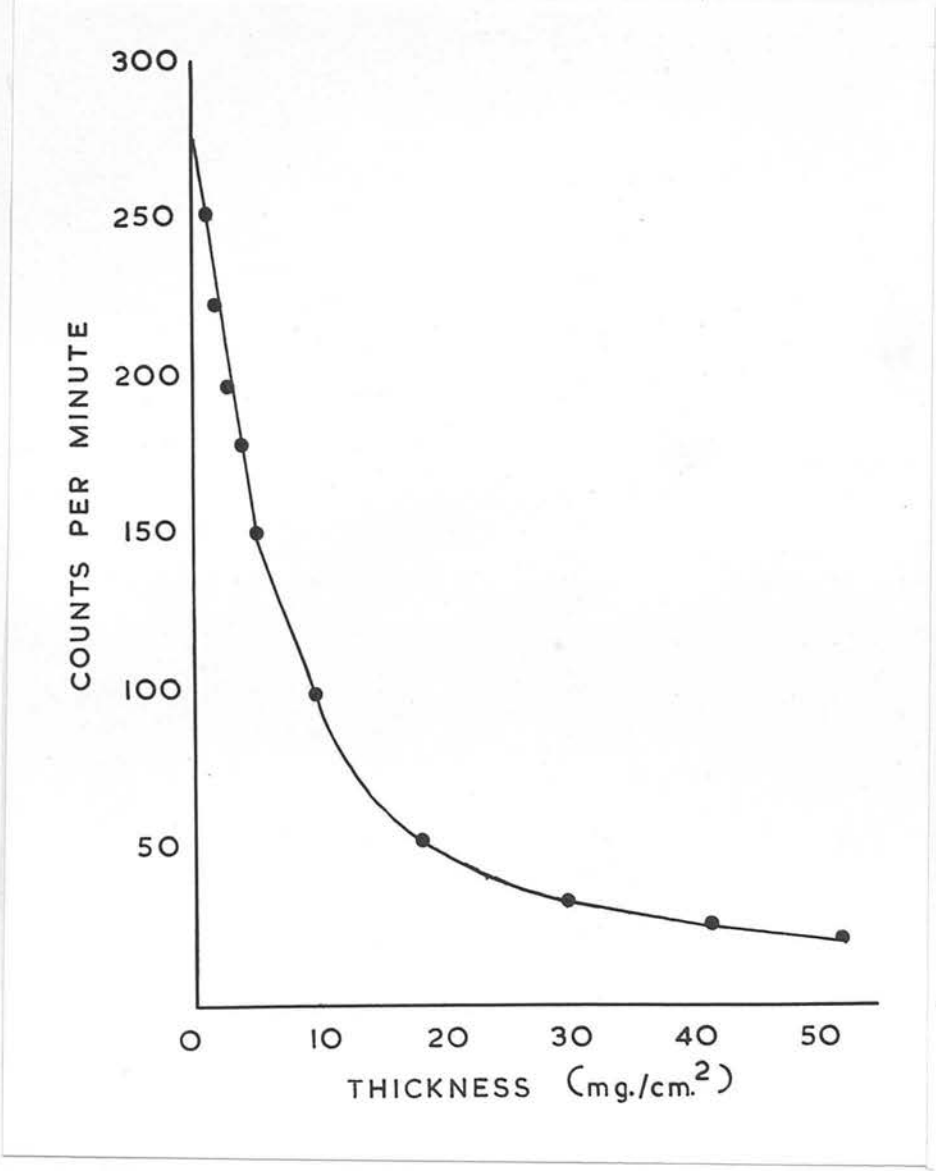

Fig. V. 3. The self-absorption curve of $Ba^{14}CO_3$ for the automatic counter (Nuclear, Chicago). The infinite thickness of $Ba^{14}CO_3$ is shown to be more than 50 mg/cm^2.

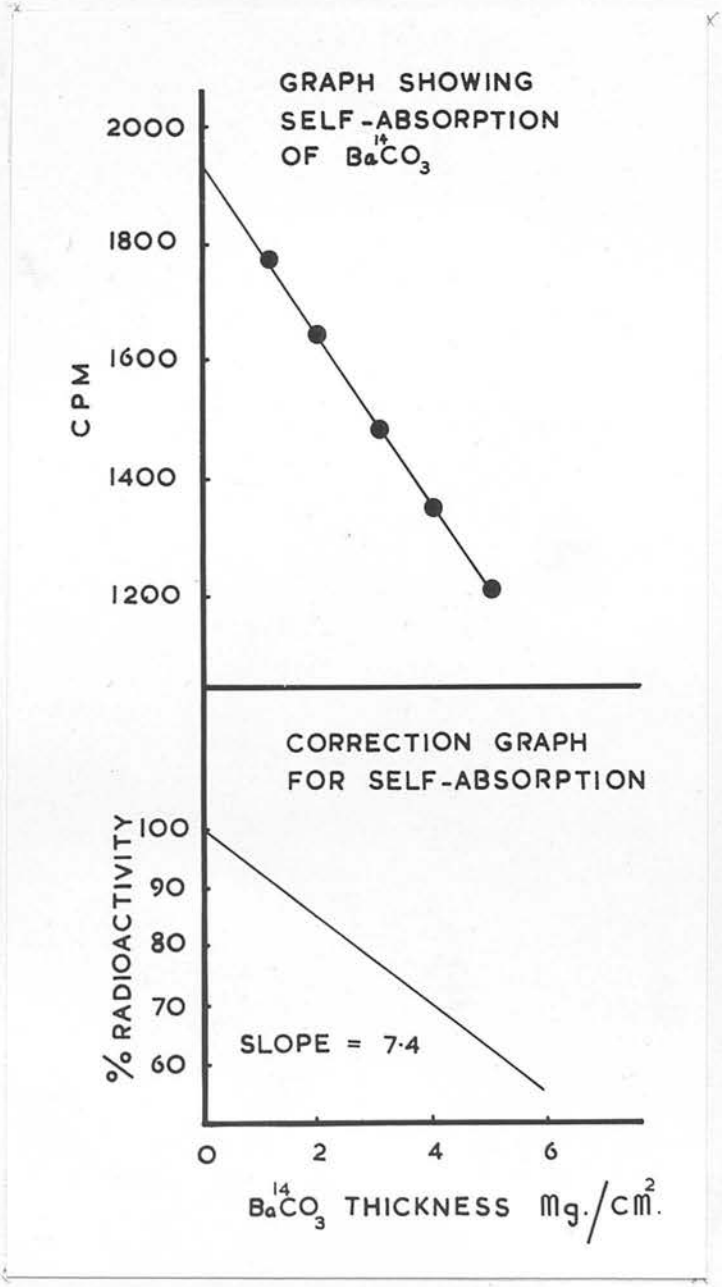

Fig. V. 4. Showing (top) self-absorption of $Ba^{14}CO_3$, the slope of which was used in constructing a correction graph for self-absorption (bottom) (see p 106).

compartment of the flasks contained weighed (100-200 mg), chopped uterine tissue in 2.5 ml of freshly prepared Krebs-Ringer phosphate solution. The side arm contained 0.3 ml of glucose solution (5 µmoles) and, when stated, other reagents (iodoacetamide, coenzyme, etc.). The gas phase was air. The oxygen consumption was followed for 1-3 hours, and results were expressed as µl per first hour per mg dry weight.

Glucose utilisation measurement

The remaining glucose in the mixture after the incubation was determined by the glucose oxidase method (Huggett & Nixon, 1957) with reagents supplied by Boehringer, Mannheim, Germany. Briefly, 1 ml of incubation mixture was added to 3 ml of 4% perchloric acid, and the precipitated proteins were centrifuged down and discarded. A 0.2 ml volume of the supernatant was then mixed with the 'glucose reagent' (contained glucose oxidase, peroxidase and o-dianisidine hydrochloride, in phosphate buffer, pH 7) and, after 35 min. standing at room temperature, the brown colour developed was measured at 436 mµ, using a Unicam SP500 spectrophotometer. The amount of glucose in the solution was then calculated from a calibration curve prepared with each set of determinations. Glucose

utilisation was then obtained by subtracting the remaining glucose quantity from the starting value. It was expressed as μmoles glucose used per hour per g. dry weight.

RESULTS

(i) Determination of the rates of glucose and oxygen utilisation in the rat uterine tissue.

The rates of glucose and O_2 utilisation by uteri of ovariectomised untreated rats and of similar animals after 4 daily subcutaneous injections of 10 µg oestradiol were determined. Results summarised in Table V. 1 show that the rates of both glucose and O_2 utilised in uteri from oestrogen treated animals are increased over the control values by 37% and 239% respectively.

(ii) The oxidation of labelled glucose by rat uterine tissue.

Results summarised in Table V. 2 show that C-1 glucose is oxidised to CO_2 at a slightly faster rate than C-6, in the uteri from all types of animals investigated. Generally, the shorter incubation periods (1 hour) give higher C-1/C-6 quotients than the longer periods (3 hours). However, the differences are only small and it is shown in Fig. V.5 that $^{14}CO_2$ production increases, after the initial time lapse, directly with the time of incubation.

It may be noted from Table V. 2 that C-1/C-6 quotients per uteri from untreated ovariectomised control

Table V. 1.

The rates of glucose utilisation and oxygen consumption in the uteri from ovariectomised rats with and without oestradiol injection. The incubation mixtures contained 100-200 mg of chopped uterine tissue, 5 µmole of glucose, 2.5 ml of Krebs-Ringer phosphate solution, pH 7.4 Results shown are mean values from two separate determinations. Ranges of the differences from means are also shown.

Types of rats	Glucose Utilisation		O_2 consumption	
	µmole/3 hr/g dry wt.	%	µl/hr/mg dry wt.	%
OVX*	65.9 ± 4.9	100	1.21 ± 0.03	100
OVX + OD** daily for 4 days	90.0 ± 4.9	137	4.10 ± 0.17	339

*OVX = ovariectomised

**OD = oestradiol

Table V. 2.

$^{14}CO_2$ recovered from the incubation of 5 µmole of either glucose-1-^{14}C or glucose-6-^{14}C with 100-200 mg of chopped uterine tissue suspended in 2.5 ml freshly prepared Krebs-Ringer bicarbonate buffer, pH 7.4. The gas phase was O_2:CO_2-95:5. Results shown are means (expressed as c.p.m. at zero thickness per mg dry weight) with S.E.M.

Types of rats	1 hr. incubation period				3 hrs. incubation period			
	No.of Expt.	C-1***	C-6	C-1/C-6	No.of Expt.	C-1	C-6	C-1/C-6
'Normal'	2	91 ± 31	38 ± 4	2.4	5	220 ± 20	112 ± 19	2.0
OVX*	5	57 ± 6	37 ± 6	1.5	5	166 ± 29	125 ± 16	1.3
OVX + OD** daily for 3 days	6	73 ± 16	39 ± 5	1.9	4	281 ± 18	168 ± 16	1.7
OVX + OD daily for 4 days	3	77 ± 2	44 ± 5	1.8	5	248 ± 28	154 ± 18	1.6

*OVX = ovariectomised

**OD = oestradiol

***C-1, C-6 denote $^{14}CO_2$ from the oxidation of C-1 and C-6 of glucose molecules respectively.

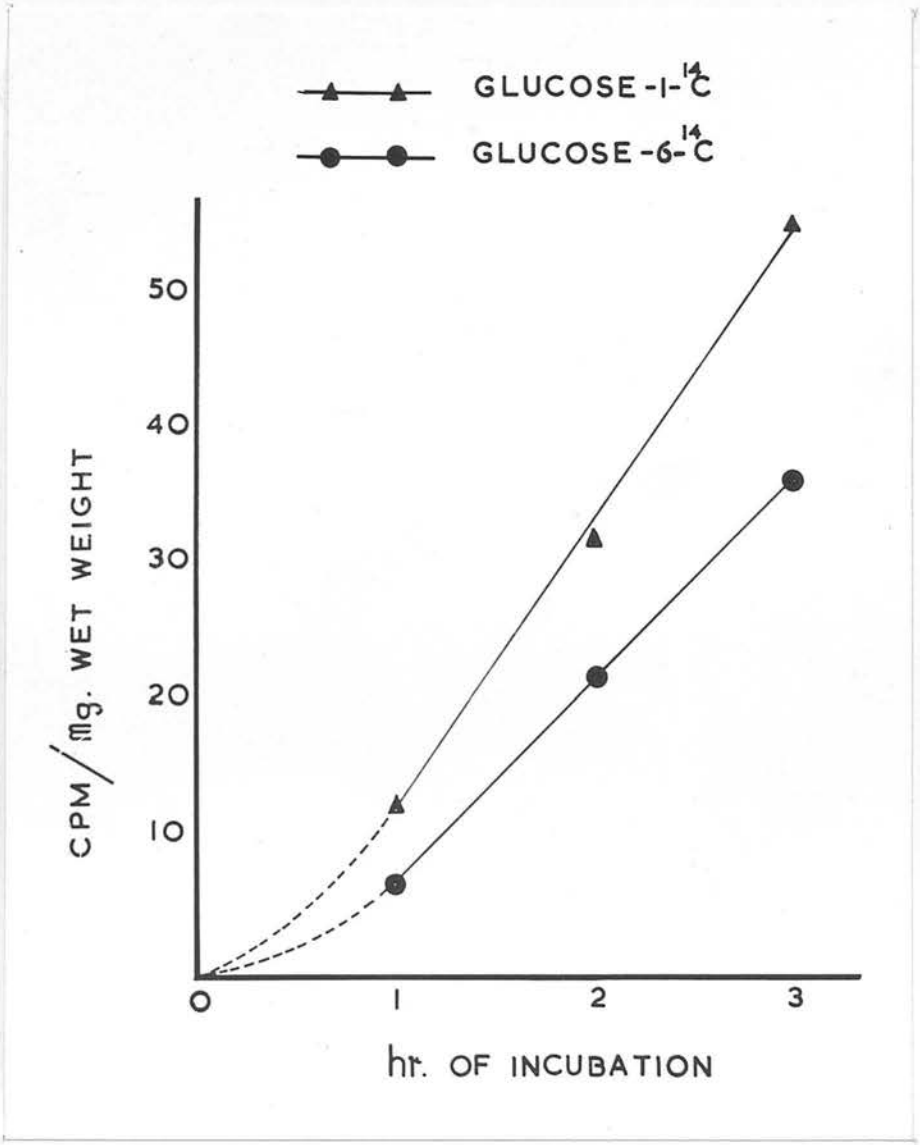

Fig. V. 5. The relationship between the time of incubation and $^{14}CO_2$ liberated from the oxidation of glucose (labelled with ^{14}C at either C-1 or C-6) by chopped rat uterine tissue. Similar results were obtained with uteri from either 'normal' or castrate rats, with and without oestrogen treatment.

rats are the lowest, compared with other results. Uteri from the oestrogen-treated ovariectomised animals appear to show an increase of 20-40% in the overall oxidation of both C-1 and C-6 of glucose as compared with the controls. However a considerably faster rate of increase is observed in the oxidation of C-1 as compared with C-6. As a result of this, higher C-1/C-6 quotients are obtained with those oestrogen treated ovariectomised rats compared with the controls.

The quotients observed with the uteri from 'normal' rats are slightly higher than those from oestrogen treated rats, again as a result of higher oxidation of C-1 than of C-6 of glucose molecules. It must be pointed out that those results should only be regarded as an indication of a trend because they are not statistically significant.

(iii) In vitro effects of oestradiol, phenazine methosulphate, insulin and iodoacetamide, on the uterine oxidation of labelled glucose.

Results summarised in Table V. 3 show that in the 'normal' rat uteri, oestradiol suppresses the oxidation of both C-1 and C-6 of glucose to about the same extent. The degree of suppression depends on the concentration of the oestrogen in the mixture. It is also

Table V. 3.

Effects of oestradiol, phenazine methosulphate, insulin and iodoacetamide on the amount of $^{14}CO_2$ recovered from the incubation of labelled glucose with rat uterine tissue. Reaction mixtures are the same as described in Table V. 1. Results are expressed as percentages of control.

Substances added to incubation mixture	'Normal' rats		OVX* rats		OVX rats + OD** daily for 4 days	
	C-1***	C-6	C-1	C-6	C-1	C-6
nil - control	100	100	100	100	100	100
3×10^{-5}M oestradiol	79	90	-	-	-	-
20×10^{-5}M oestradiol	47	39	-	-	-	-
10^{-4}M phenazine methosulphate	-	-	980 -	166 -	861 966	217 248
Insulin (1 i.u./2.5 ml)	-	-	132 109	131 120	130 133	107 147
10^{-4}M iodoacetamide	2.1 -	4.1 6.5	-	-	-	-

*OVX = ovariectomised

**OD = oestradiol

***C-1, C-6 denote $^{14}CO_2$ from the oxidation of C-1 and C-6 of glucose molecules respectively.

shown that in the uteri of similar animals, iodoacetamide at the concentration chosen inhibits the glucose oxidation almost completely (93-98%). The inhibitions of oxidation at C-1 and C-6 by iodoacetamide are probably equal. In uteri of castrate rats, either with or without oestrogen treatment, phenazine methosulphate stimulates C-1 oxidation 9-10 times and C-6 oxidation by about twice. On the other hand, in tissues from similar animals insulin appears to stimulate the oxidation of C-1 and C-6 to a small extent (20-30%).

DISCUSSION

It has been demonstrated in the present investigation (Table V. 1) that uteri removed from castrate rats 4 days after the start of oestradiol injections show increases in the rate of glucose (determined enzymically) and oxygen consumption, as compared with the controls. The increases are similar to those observed up to 24 hours after oestrogen administration (Roberts & Szego, 1953). The increase in glucose metabolism is also observed in measurements made with radioactive labelled glucose. Thus, the radioactivity of $^{14}CO_2$ recovered from the incubation of uterine tissues obtained 3-4 days after oestrogen administration is higher than that observed with uteri of similar animals not receiving oestrogen. Similar results are obtained by both methods.

The fact that the uteri of rats treated in different ways gave a C-1/C-6 quotient not differing greatly from unity, and that iodoacetamide almost completely inhibits glucose oxidation, indicates that glycolysis is the main catabolic pathway of glucose in the rat uterus. Nevertheless, some hexose phosphate 'shunt' activity is indicated even in the uteri from ovariectomised rats (which show the lowest C-1/C-6 ratio), since the oxidation of C-1 of glucose is faster than of C-6 (Table V. 2). The

slightly lower C-1/C-6 quotients obtained when the incubation time is prolonged may be due to a more extensive recycling process in the 'shunt' pathway during the longer incubation. Similar observations have been reported in the study with thyroid tissue (Dumont, 1960).

The finding that the oxidation of C-1 of glucose is increased more than of C-6 in uteri from oestrogen-treated castrate rats, although only of limited significance, may indicate a tendency to increase the 'shunt' activity in the oestrogen treated tissue. The fact that phenazine methosulphate stimulates the oxidation of C-1 of glucose to a much greater extent than of C-6 shows that the re-oxidation of TPNH may be an important factor in controlling the activity of the 'shunt.' Since TPNH is likely to be utilised in vivo in the growing uteri of oestrogen-treated castrate rats more than in the atrophic control uteri, one can imagine more glucose being metabolised via the 'shunt' pathway in the first case than in the latter. The small increase in the 'shunt' activity in the uteri of oestrogen treated animals (as seen from the increase in the C-1/C-6 quotient observed in vitro) may thus be of more significance in vivo where biosynthetic processes are proceeding at an optimum pace. The in vitro incubation, as done in this investigation, with glucose as the sole substrate, may not

represent the right conditions for a substantial rate of biosyntheses. It is therefore possible that the uteri of oestrogen treated animals metabolise glucose via the hexosemonophosphate pathway to a greater extent *in vivo* than is indicated by the *in vitro* measurements now reported.

Insulin appears to stimulate the oxidation of both C-1 and C-6 to about the same extent, indicating that this hormone promotes glucose metabolism by the two pathways, either to the same degree, or at an early common stage of the two routes, as for example by increasing cell permeability to glucose or by activating hexokinase (see Fisher, 1960). The ability of insulin to stimulate the C-1 oxidation in the mammary glands from lactating animals (but not in the glands from pregnant rats) (McLean, 1959), is attributed to the fact that the hormone increases the *in vitro* synthesis of fatty acids from glucose (Balmin, Folley & Glascock, 1954; Abraham, Cady & Chaikoff, 1957). However, since insulin does not stimulate the oxidation of C-1 of glucose in uteri from rats receiving oestrogen to a greater extent than in the uteri from the untreated animals, this may indicate either the inactivity of hexosemonophosphate shunt in the uterus, or the inability of insulin to stimulate the TPNH - reoxidising system in this tissue. It may be pointed out that the biosynthetic processes in

the lactating mammary glands, where milk production is predominant, and in the proliferating rat uterus are not of the same nature, and may not be influenced by insulin in the same fashion.

It is interesting that there is only a slight tendency to increase the activity of the hexosemonophosphate 'shunt' in uteri from castrate rats 3-4 days after the oestrogen administration, in spite of the very marked increases in the levels of G6PDH and 6PGDH (see SECTION IV). It is known, however, that increases in these two enzymes may not necessarily be accompanied by a corresponding rise in the 'shunt' activity, and an increased participation of glycolysis may even be obtained. For instance, the levels of both G6PDH and 6PGDH in the liver of thyroxine-treated rats are increased significantly (Glock & McLean, 1955c), yet only the glycolytic pathway is found to be stimulated; the 'shunt' activity appears to be unaffected (Glock et al, 1956b). It is not yet know whether there is any increase in the level of phosphoglucose isomerase (the enzyme in the glycolytic pathway which competes with G6PDH for glucose-6-phosphate) in the uteri from castrate rats after a similar period of oestrogen administration. Information concerning this enzyme may be critical because the increase in the level of phosphoglucose isomerase could counteract most of

the increase in the level of G6PDH and result in only a small overall increase in the 'shunt' activity. Glucose-6-phosphate can be metabolised in a number of different ways, and it has been shown, with liver slices, that the pattern of metabolism is influenced by changes in cationic concentrations in the incubating medium, or by using liver from diabetic rats (Ashmore, Cahill, Hastings & Zottu, 1957). A similar comprehensive investigation of the effect of oestrogen on the pathways of glucose metabolism in the rat uterus may thus be in order.

SECTION VI

GENERAL DISCUSSION

In the present investigation, two main approaches were made in a study of the mechanism of action of steroid hormones, on the assumption that the hormones produce their biological effect either by participating in, or by specifically affecting some enzyme systems.

The first approach is an attempt to obtain further support for the postulation of Villee and Talalay that a transhydrogenase, such as that found in human placenta, is the locus of steroid action.

Before assessing the problem in the light of the new findings reported in this thesis, let us survey the merits of the transhydrogenase theory. The most novel aspect of this theory is that it offers a very clear cut explanation of the effect of oestrogenic hormone added in low concentration (10^{-8} - 10^{-6}M) to an <u>in vitro</u> system. The concentration of hormone used is within the range that one might conceive to be present in the tissues (see Engel & Scott, 1960). Furthermore, the effects of various oestrogens on the placental transhydrogenase are roughly parallel to their biological activities. Oestradiol and oestrone have a strong stimulating effect, oestriol possesses a much weaker effect, and oestradiol-17α and many other steroids are completely devoid of effect on the transhydrogenase system. The apparent lack of effect on

transhydrogenase of potent synthetic oestrogens such as diethylstilboestrol, was a serious objection to the validity of this theory. It has recently been shown, however, by Glass, Loring, Spencer & Villee (1961) that these substances do in fact stimulate the enzyme. Very low concentrations of stilboestrol must however be used, because concentrations higher than 3.7×10^{-6} M cause inhibition. The failure of the earlier attempts were due to the use of too high concentrations. Another merit of the theory is that it enables one to visualise the role played by oestrogen in its specific and controlling effect upon metabolic processes (see p. 7). These observations are consistent with the view that the effect of oestrogen on transhydrogenase can explain their biological activity but are by no means proof that this is the case.

The most disturbing feature of Villee's transhydrogenation theory is the apparent absence of the oestrogen-sensitive transhydrogenase activity in the known 'sites' of action of oestrogen such as in the human endometrium, rat uterus and vagina and chicken oviduct, etc., as shown in SECTIONS II and III of this thesis. An endogeneous inhibitor may be present in these tissues (p. 42), and this may prevent the in vitro demonstration of transhydrogenase activity, but one fails to see any

physiological importance of the transhydrogenation system to the cells concerned under such a condition. It is of course quite possible that this inhibitor does not come into effect *in vivo*. Indeed one of the most important tasks for the future is to examine the nature of this inhibition. In view of the fact that similar negative results are obtained when both *in vitro* (SECTION II) and *in vivo* (SECTION III) techniques are employed in the investigation of transhydrogenation in the rat uterus, it seems unlikely that the failure to demonstrate transhydrogenase activity is due to inappropriate experimental conditions. It is more probable that the enzyme is absent from the tissues.

Apart from the human placenta, the oestrogen-activated transhydrogenase has been shown only in one other 'target' organ, namely the human breast (Hollander *et al*, 1958). But even in this tissue, only 46.9 per cent of the specimens examined by Hollander (1958) showed sensitivity towards oestrogen. A fact of this kind is unexpected, since, unless some other limiting factor is operating, all normal breast tissue should be sensitive to oestradiol. It is difficult to imagine a key enzyme to be present in less than half of the samples examined while it is known that breast tissue invariably responds to oestrogen

stimulation.

The physiological significance of the presence in the human placenta of an oestrogen-sensitive transhydrogenase, can be questioned on the grounds that the placenta is hardly a typical 'site' for oestrogen action. There is no particular evidence that an oestrogen plays a physiological role in placental tissue as it does in more characteristic target tissues. During the major part of the life span of a woman, the placenta does not exist in her body and may never do so if she is not to bear a child, although she will enjoy all the feminine characteristics, reflecting the normal functioning of her oestrogenic hormones. The placenta arises partly from the endometrium, and partly from the foetus. Its growth parallels that of the foetus, and it is expelled from the maternal body at term. It is not too far from fact to state that the placenta, like the foetus is 'external' to the maternal body. It is therefore perhaps unwise to look to a phenomenon occurring in a somewhat external tissue as a model for the mechanism of action of oestrogen in the normal female organism, especially when the evidence for this certain phenomenon occurring in most of the usual sites of oestrogen in the body cannot be obtained.

The oestrogen-sensitive transhydrogenase,

found in the soluble fraction of human placenta and breast tissue, may, as suggested by Mueller (1960, 1961) be induced in response to the abnormally high levels of endogenous oestrogen supplied to these tissues. This elevated supply of oestrogen is certainly true for the placenta and may also be true under certain circumstances for breast.

Since the known oestrogenic steroids produce similar responses in most species, their *in vitro* effect should not be species-specific. The evidence obtained in SECTION II, shows that oestrogen-sensitive transhydrogenase does not meet this criterion. Among the placentae from 6 mammalian species investigated, only that from human subjects is found to contain this enzyme. This further stresses the danger of attempting to generalise effects of hormones between, not only different tissues, but also similar tissues from different species.

With regard to the quantitative significance of the human placenta and breast transhydrogenase, even at the maximum oestrogen stimulation the enzymic activity is much lower (about a tenth) than the mitochondrial transhydrogenase (Villee *et al*, 1960) of the same tissue, whose activity is already small compared with similar enzyme activity in some tissues such as heart (Humphrey, 1957).

Stein & Kaplan (1959) have in fact suggested that the term transhydrogenase be reserved for the more active mitochondrial enzyme. Although the suggestion is rather unfair in view of the true transhydrogenation catalysed by the placental soluble enzyme, the difference between the activities of the two enzymes remains striking. As Villee et al (1960) point out, if the role of transhydrogenase is to produce DPNH, the oxidation of which is coupled with phosphorylation to yield ATP, then the soluble enzyme enjoys the more abundant supply of TPNH, since the two major TPN reducing enzymes G6PDH and ICDH are located in the soluble part of the cytoplasm. However, this advantage, due to cellular compartmentation, is rather doubtful in view of the fact that the oxidation of DPNH occurs in the mitochondria. Moreover, under the most favourable conditions produced in vitro transhydrogenation catalysed by the soluble human placental enzyme is much weaker than that effected by the mitochondrial enzyme. The physiological significance of this weak transhydrogenation, for energy production is very questionable when a more powerful enzyme system is available.

Thus it appears that in spite of the attractive aspects of the transhydrogenase theory for the mechanism of action of oestrogens, it is not without drawbacks. It may

be pointed out here that these difficulties are equally true whether we accept Villee's view that oestrogens "activate" the transhydrogenase in some manner unknown or Talalay's that the oestrogens act as coenzymes (p. 6, see also p. 44).

Although the transhydrogenase theory cannot, at present, be accepted as the answer to the problem of mechanism of oestrogen action, the idea of oestrogen influencing some enzyme can still be regarded as an attractive working hypothesis. Thus it is possible that the 'target' organs, by embryonic differentiation differ from the 'non-target' organs in one or more enzymes, with unique responsiveness to hormonal stimulation. As a result of this stimulation, key metabolites may accumulate and promote or induce other enzymic reactions (common to both 'target' and 'non-target' tissues) leading to characteristic responses such as growth. It is thus possible to have characteristic responses at the 'site' of action of a hormone, but not in other tissues. The identification of such an enzyme (or enzymes) in endometrium, uterus and pituitary (see p.40), which respond to oestrogens added *in vitro* by an increase in oxygen consumption by those tissues, would be interesting because of the possibility that it may prove to be the specific enzyme

discussed above.

The second approach to the study of the mechanism of action of oestrogenic steroid hormone, chosen in the present investigation, involves the pretreatment of the experimental animals with the hormone, and subsequent removal of a suitable 'target' tissue for comparison of its biochemical reactions with those of the tissue from an untreated animal. Since the system employed here, unlike the single enzyme transhydrogenase already referred to, is very complex and cannot be readily defined biochemically, the differences between stimulated and unstimulated tissues, when obtained, may be due either to a primary or a secondary effect of the hormone concerned. However, information obtained from such a study should be of value in singling out sequences of biochemical reactions influenced by administered oestrogen, and by tracing these back in time it might well be possible to identify the locus of the initial action of the hormone. The biochemical parameters chosen for study here - namely the concentrations of oxidised and reduced pyridine nucleotides, the levels of pyridine nucleotide-linked dehydrogenases and the metabolic pathways of glucose in the rat uterus, represent only a few aspects of the biochemical activities of the cell. Nevertheless, the results obtained have

certain features in common with those from a similar study of different enzymic systems by Mueller. Thus marked increases in the levels of G6PDH, 6PGDH and LDH in the uteri from oestrogen treated rats (SECTION IV) are considered to increase the production of DPNH and TPNH (p.p. 66 and 91), presumably required in the stimulated anabolic processes concerned with the synthesis of protein, nucleic acids and lipids occurring in the proliferating uterus. (Mueller et al, 1958; Mueller, 1960 and 1961). This view is supported by the fact that the concentrations of DPNH and, to some extent, of TPNH increase at the same time as the increases in the levels of the dehydrogenases (SECTION III). The increases in the oxygen and glucose utilisation by the stimulated uteri, including a tendency to increase the glucose catabolism via the hexosemonophosphate 'shunt', also indicate an increase in the supply of energy and intermediates (e.g. ribosephosphate) to the growing tissue. These observations are in accord with the view that oestrogen is affecting the uterine cells by modifying the environment in such a way that many biosynthetic processes are facilitated with the resultant discernible uterine growth (Fig. I. 1). It may be noted that the stimulation of these biosynthetic processes are probably brought about by the increases in the levels of the enzymes responsible, as distinct from a

permeability phenomenon. In SECTION IV (p. 90) some evidence is presented that these increases are due to an increase in the amount of enzyme protein, synthesised de novo, rather than to stimulation of pre-existing enzyme. In recent reviews of their studies on the mechanism of action of oestrogens Mueller et al (1958), Mueller (1960 & 1961), arrive at a similar conclusion. This is based on two main facts: the inability to activate pre-existing enzyme molecules by oestrogen added in vitro, and the failure to stimulate biosynthesis of nucleic acid purines and pyrimidines and of lipids when protein biosynthesis is blocked by puromycin (see Yomolinski & De la Haba, 1959).

It is interesting that oestrogenic hormones are not the only ones in the steroid world whose mechanism of action is connected with stimulation of de novo synthesis of enzyme protein. Thus Umbreit (1951), who studied the influence of cortisone on proline oxidase in kidney and D-amino acid oxidase in liver, came to the conclusion that the effect of cortisone is apparently upon the amount of enzyme present rather than the activation of the hormone. The fact that ethionine (a substance known to interfere with protein synthesis by competing with methionine) prevents the increase in the level of tryptophan peroxidase in the rat liver induced by the administration of cortisone

led Knox & Auerbach (1955) to believe that the increase is resultant from the rise in the amount of newly synthesised enzyme protein. The increase in the level of β-glucuronidase in the kidneys of mice treated with androgens is found by Fishman (1961) to be due to an increase in the amount of enzyme protein, based on the incorporation of radioactivity from glycine-1-^{14}C administered to animals undergoing androgen administration. Indeed, in the most recent review on the mechanism of action of androgens, Dorfman (1961) proposes that androgens act by the stimulation of the rate of biosynthesis of specific enzyme protein. It thus appears that, although the steroid hormones may differ vastly in their biological activity, their mechanism of action may prove to be similar in nature.

Thus it appears that one of the very first actions of oestrogen is to 'trigger' off the machinery for synthesising specific enzyme protein. At present, just how oestrogen brings this about, and why the stimulation occurs only in the 'target' organ, are matters for speculation. Mueller suggests that the hormone might, somehow, remove membranous 'barriers' which cover the templates (consisting of a combination of enzyme protein and RNA) involved in the enzyme synthesis. It is also

necessary to postulate that, due to embryonic differentiation established in each type of tissue, only the 'template barriers' in the 'target' organ are capable of responding to oestrogenic hormones. It may be noted that, this speculation is different from the principle involved in the transhydrogenation theory (p. 122) in that, instead of having a completely new enzyme in the 'target' organ, there are the same sets of enzymes in all tissues. They are, however, different quantitatively and qualitatively in various cells, giving rise to a different response in different cells. There is evidence indicating that there may be a fundamental difference between the 'target' and 'non-target' organs. Thus, on injection of tritium-labelled oestradiol into young female rats, radioactive oestrogenic steroids appear in and are retained by the uterus, vagina and pituitary gland longer than those in the liver, kidney, muscle, adrenal, blood and bone (Flesher, Gupta, Jacobson & Jensen, 1960). In a similar investigation, oestradiol alone is found in the rat uterus, but mixtures of conjugated steroids are found in liver (Saha, Flesher, Jacobson & Jensen, 1960). It thus appears that there is a selective localisation of exogenous steroid in 'target' organs.

In view of the diverse physiological responses

described in a variety of species following in vivo administration of oestrogen (p. 1), it is possible that the mechanism of action of this hormone may not necessarily be limited to only one. The importance of the compartmental nature of the cells for organised functions has been realised (Siekevitz, 1959) and it is also known that some hormones affect cell membranes in such a way that substrate or cofactor molecules are more readily available to the enzyme and thus increase the overall rate of the specified enzyme system. Insulin, for instance, is well established that its effect on sugar permeability is primary to an effect of insulin on glucose uptake and metabolism (see Levine, 1957). Beside the enzymic approach to the study of the mechanism of action of oestrogen, work has also been done on the effect of oestrogen on the permeability of the uterine cells. Hechter & Lester (1960), who measured the distribution of radioactively labelled inulin, sucrose, D-xylose, α-aminoisobutyrate, ^{22}Na and ^{86}Rb between tissue and cell water, using uterine tissue from ovariectomised rats with and without oestrogen treatment, found that there are no striking differences in cellular permeability to these substances between the two types of uteri. These workers conclude that oestrogen does not affect the uterine cell membrane to facilitate substrate

entry, although they have not ruled out the possibility that oestrogen might influence the permeability of only a limited number of cells in the uterus. In this connection, it is also possible that the hormone might alter the permeability of sub-cellular bodies such as of mitochondria.

In conclusion, it appears that there is no evidence available at the present time to permit conclusions regarding the primary physical or chemical events at the cellular level, which are influenced or regulated by oestrogenic hormones, or any other steroid hormones. However, the author feels, the future is promising.

SUMMARY

The mechanism of action of steroid hormones, in particular of oestrogens, has been investigated along two main lines.

(a) Attempts have been made to assess the physiological role of an oestrogen-sensitive transhydrogenase first demonstrated in the placenta of women. The original observations have been confirmed but it has not been possible to demonstrate the same enzyme activity in placentae from rat, guinea-pig, rabbit, golden-hamster or pig. Nor has the enzyme been found in such oestrogen "target" organs as human endometrium, rat and guinea-pig uterus, rat vagina, chicken oviduct or ox pituitary. Negative results are not due to destruction of substrates or coenzymes during the enzyme determinations, but may be due to the presence of endogeneous inhibitors.

The ratios of oxidised to reduced di- and triphosphopyridine nucleotides have been determined in the uteri of intact, ovariectomised and ovariectomised oestrogen treated rats. Changes in these ratios in response to oestrogenic steroids give no indication of transhydrogenase activity *in vivo*.

The inability of some 3β- and 11β-hydroxy steroids to act as coenzymes and promote transhydrogenation

in the liver and adrenal has also been observed.

(b) Changes in levels of glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconic acid dehydrogenase (6PGDH), isocitric dehydrogenase (ICDH) and lactic dehydrogenase (LDH) and in concentrations of oxidised and reduced di- and triphosphopyridine nucleotides in the ovariectomised rat uterus in time in response to oestradiol-17β have been investigated. The concentrations of the oxidised pyridine nucleotides remain unchanged, but the reduced forms of the coenzymes decrease in concentration up to 6 hours after oestrogen administration and then show a steady increase until at 72 hours the concentrations are significantly higher than in the uteri from ovariectomised controls.

The levels of uterine G6PDH, 6PGDH and LDH are increased by oestrogen. That of ICDH shows no change.

It is suggested that there is an overall increase in reduced coenzyme concentration due to the increased levels of enzymes concerned but that the initial decrease in reduced coenzymes may be related to demands for energy and specific reductants for biosynthesis in response to oestrogen.

Some evidence is produced to support the view that the increases in levels of enzymes are due to synthesis

of new enzyme protein rather than to activation of existing enzyme.

Changes in concentration of G6PDH and 6PGDH but not in ICDH suggested the possibility of an effect of oestrogen on glucose catabolism. This has been investigated using ^{14}C-labelled glucose. In general oestrogen is found to stimulate glucose catabolism, the increase being mainly via the hexosemonophosphate 'shunt' pathway. Glycolysis is however the major pathway of glucose metabolism in the uteri of ovariectomised and oestrogen treated rats.

It has also been shown here that the use of the 'shunt' pathway is dependent on the rate of reoxidation of reduced triphosphopyridine nucleotide. Since this reoxidation may proceed at a greater rate in vivo than under the in vitro conditions of the present experiments, the 'shunt' pathway may in fact be of greater significance than it has been possible to demonstrate in this investigation.

The probable mechanisms of action of oestrogenic hormones are reviewed in the light of the results now obtained. It is concluded that the transhydrogenase theory of Villee & Talalay is of doubtful importance in explaining the action of oestrogens. It also

seems likely that oestrogenic hormone is concerned with the induction of the biosynthesis of new enzyme protein and that this process in some way 'triggers' the whole anabolism of the 'target' tissue.

APPENDIX I

Chemicals used

DPN, TPN, DPNH, TPNH and yeast ADH were obtained from Boehringer & Sons, Mannheim-Waldhof, Germany. The DPN was assayed by the method of Racker (1950), and found to be 94% pure and contained no detectable amount of TPN. TPN, DPNH and TPNH were assayed fluorometrically against standard DPN solution from above (see Bassham et al, 1959).

G6P (di-potassium salt), 6PG (sodium salt), ATP (di-sodium salt), G6PDH (practical type II) and phenazine methosulphate were obtained from Sigma Chemical Co., St. Louis, U.S.A.

Oestradiol-17β, oestriol, oestrone, oestradiol-17α, progesterone, cortisol, cortisone, DOC-acetate, pregnenolone, testosterone, androsterone were obtained from the Medical Research Council Steroid Reference Collection, or from Messsrs. Organon Laboratories Ltd., Newhouse, Lanarkshire.

2-fluoro-oestradiol-17β was a gift from Dr. G. C. Mueller, University of Wisconsin Medical School, Madison, U.S.A.

MER-25 was a gift from Messrs. Wm. S. Merrell & Co., Cincinnati, U.S.A. Amphenone-B was a gift from the The Upjohn Company, Kalamazoo, Michigan, U.S.A.

DON was a gift from Park Davis & Co.

Crystalline insulin (6 X recrystallisation) was obtained from Boots Pure Drugs Co., Nottingham, England.

APPENDIX II

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APPENDIX III

PUBLICATION

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(11) Effect of Oestradiol-17β on the Production of Reduced Triphosphopyridine Nucleotide in the Rat Uterus.

By K. MONGKOLKUL and J. K. GRANT.* (*Department of Biochemistry, University of Edinburgh*)

The importance of triphosphopyridine nucleotide in the reduced form (TPNH) in reductive biosynthesis is now generally recognized. The production of TPNH in the oestrogen-stimulated rat uterus has therefore been studied.

In the present investigation no significant increase in concentration of TPNH in the ovariectomized rat uterus could be demonstrated after administration of oestradiol-17β *in vivo*. Such an observation does not, however, preclude an increased rate of turnover of TPNH as might be indicated by increased activity of TPNH-generating systems. No evidence has been obtained that increased amounts of TPNH arise from stimulation of a pyridine nucleotide transhydrogenase by oestrogen, although this effect has been demonstrated in human endometrium and other tissues (Hagerman & Villee 1952; Talalay & Williams-Ashman, 1958).

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It has been found, however, that the glucose 6-phosphate and 6-phosphogluconic acid dehydrogenase activities of the uterus *in vitro* are markedly enhanced by administration of oestradiol-17β to castrated rats. This effect is diminished by the simultaneous administration of the cytotoxic drug DON (6-diazo-5-oxo-nor-L-leucine) which is known to inhibit uterine growth in response to oestrogen (Poulson, Robson & Wander, 1960). The activity of *iso*citric dehydrogenase is uninfluenced by oestrogen administration. It is therefore possible that an important effect of oestrogen in the uterus is to control glucose oxidation by the pentose phosphate shunt and thus to influence the availability of TPNH and to provide ribose 1-phosphate for nucleoside biosynthesis at the same time.

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